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CHARACTERIZATION OF A COLD TEMPERATURE SENSITIVE MUTANT OF ARABIDOPSIS

by

Ⓒ PETER J. McCOURT

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
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Date...September 29, 1982.....

ABSTRACT

A mutant (*colD1*) of *Arabidopsis thalania* which is sensitive to chilling temperatures(13° - 16°C) was isolated and partially characterized. Temperature shift experiments demonstrate that the lethal effect of chilling is irreversiable after a four day exposure. The mutant shows an increased dark respiration rate and progressive loss of photosynthesis over this time period.

Preliminary analysis using $^{14}CO_2$ and ^{14}C -Acetate feeding experiments suggest the mutation causes alterations in lipid metabolism. All other areas of carbon metabolism appear to show normal product distrubution. The alterations in lipid metabolism are not reflected in the unsaturation/saturation levels suggesting that the changes at the total lipid pool level are small and cannot be detected by gas chromatography of total leaf fatty acids.

The changes in lipid metabolism could be responsible for the loss of the ability of the mutant to maintain fluid membranes at chilling temperatures.

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I. INTRODUCTION

Genetic variabilty associated with response to temperature can be observed at many levels of organization in the plant kingdom. This diversity has probably evolved as a result of the various microclimates associated with this planet. In general, plants have adapted to a limited range of enviromental conditions. If one of the conditions change beyond the range of adaption, the plant becomes stressed.

For example, while many species normally found in tropical and subtropical regions are injured by low temperatures above 0°C, numerous temperate zone species can withstand freezing temperatures. Plants may, therefore be artifically subdivided into cold hardy and cold sensitive species. *Cold sensitive* plants are those which are killed or seriously injured by temperatures which may range from just above the freezing point of the tissue up to 15 - 20°C in some cases. An extreme example in this regard is *Tidestromia oblongifolia*, a shrub from Death Valley California, which has an optimum temperature of photosynthesis of 45°C. At 20°C or less photosynthesis stops and dormancy is induced (Bjorkman 1980). In general, the severity of damage incurred by chilling of cold sensitive species usually increases as temperature is progressively reduced below some threshold level, and is time-dependent (Lyon *et al*,, 1979). Plants able to grow near zero degrees Celsius are classified *chilling resistant*.

There is no obligate relationship between optimal growth temperature and sensitivity or resistance to chilling. For instance potato and tomato have similar temperature optima but the former is chilling resistant whereas the the other is extremely sensitive. While considerable information has been assembled in regard to the phenomenon of chilling injury, little is known about the exact sequence and timing of events at the cellular and molecular levels. If one is ultimately going to improve plant resistance to low temperature for the purposes of agriculture and horticulture one must : (1) characterize the stress imposed on the plant; (2) determine the repercussions of stress on the cellular environment; and (3) determine what constitutes injury at the cellular and molecular level. The experimental approach to understanding these three parameters has largely taken the form of comparaitive physiology of cold hardy and cold sensitive species. However, it has been difficult to attribute specific effects to differences that are found because of the broad differences in genetic background between resistant and sensitive species. Although *Lycopersicon sp* (tomato) and *Zea mays* (corn) are evolutionarily distant, the argument is that they must share a trait or number of traits that render them chilling sensitive. On the basis of this rationale certain aspects of plant physiology have intensively studied in the hope of identifying the unifying chilling sensitivity trait or traits.

Membrane lipids

The most strenuously advanced theory of chilling sensitivity concerns the effect of temperature on the mobility of membrane lipids (Lyon, 1972). At low temperature lipid bilayers become crystalline with lipids tightly packed together. As the temperature is raised above a transition temperature the spacing increases. The bilayer continues to adhere but the fatty acids melt and are free to rotate. This flexibility of individual lipid molecules is termed fluidity and is believed to be the state suitable for life processes.

When the temperature is lowered to the transition temperature the lipid bilayer starts to change to the solid non functional state. The activity of enzymes, structural proteins and/or transporters embedded in or attached to these membranes are dependent on the fluidity of the membrane. By disrupting the activity of one or more of the membrane associated functions by a low temperature transition cellular metabolism may be abruptly altered.

Drastic changes in various physiological activities have been observed in the vicinity of these transition temperatures. For example, mitochondrial membranes from chilling sensitive plant species (Raison *et al*, 1971; Raison and Chapman 1976) and chloroplast membranes (Raison 1974) all exhibited physical phase transitions in response to temperature. The phospholipids extracted from each of these membranes undergo a phase transition as discerned by electron spin resonance at the same critical temperature observed for intact membranes and these transitions could be correlated with visual melts of defined lipid systems (Mehlhorn *et al*, 1973).

In general, the transition temperature can be expected to depend on the lipid species as well as chain length and degree of saturation of the fatty acid constituents. This is illustrated by studies of *Anacystis nidulans*, a blue green alga. In this organism the degree of saturation of membrane lipids is strongly dependant on growth temperature (Halton *et al*, 1964). As the temperature increases the degree of saturation also increases. Moreover O_2 evolution, electron transport and other indices of photosynthetic function show abrupt changes at phase transition temperatures (Murata and Folk, 1975). These studies indicate a possible causal relationship between the degree of lipid saturation, phase transitions, and photosynthetic function.

Unequivocal evidence in favour of this hypothesis has not yet been obtained in higher plants. Decreases in temperature have been shown to cause increases in the degree of unsaturation in some higher plants. Transfer of *Brassica napus* (rapeseed) to 5°C leads to increases in the level of linolenic (18:3) acid in the roots and leaves (Smolenska and Kuipia 1977). This increase was most evident in phosphatidylcholine and phosphatidylethanolamine fractions of the leaves, and in the neutral lipids and an unidentified phospholipid from the roots. In studies of roots from *Secale cereale* (rye),cooled to 8°C the relative abundance of phosphatidylcholine declined while phosphatidylethanolamine increased (Clarkson *et al*, 1980). The acyl lipids contained an increased proportion of linolenic acid (18:3) at the expense of linoleic acid (18:2). However the lipids of chilling sensitive *Ipomaea batatas* (sweet potato) have higher proportions of unsaturation than lipids of chilling resistent *Solanum andigena* (potato) (Yamaki and Uritani,1972) Compositional comparisons of phospholipids, fatty acids and sterols between seeds of *Pisum sativum* (Pea) and *Glycine max* (soybean) show few differences although germination of soybean is inhibited by the cold (Priestly and Leopold 1980). Polar lipids and their degree of unsaturation from leaves of *Passiflora* species which varied in resistance to chilling were found to be similar (Patterson 1978). Therefore, unlike the simpler systems such as blue green algae and bacteria, the whole plant analysis of cold hardiness has proven complex. This has led to attempts to subdivide the problem by examining specific effects of temperature on component physiological processes.

Dark Respiration

Research in this area has been dominated by the observation that many membrane bound enzymes associated with the mitochondria of chilling sensitive plants show nonlinear Arrhenius plots with an increase in the activation energy of the enzyme below about 10 - 12°C. The basic principle behind an Arrhenius plot is that reaction rates increase exponentially with temperature. When experimental results of these rate measurements at different temperatures are plotted as log rate versus the reciprocal of the absolute temperature, the Ea or Arrhenius activation energy can be determined from the slope of the line. The expectation for biological systems from Arrhenius theory is a linear relationship from freezing to denaturation temperatures. The existence of a "break" (discontinuity) in the slope of an Arrhenius plot can be explained by the occurrence of a phase change occuring at that temperature. In studies of oxidative activity of isolated mitochondria from chilling resistant plant species a linear plot was found with constant activation energy over a temperature range from 1 - 25°C (Lyon and Raison 1973). In contrast, the mitochondrial respiration from chilling sensitive species exhibited a discontinuity in the slope at 10 - 12°C, with a marked increase in Ea below the "break" temperature. This indicates that the intermediate or direct effect of low temperature on these species is to supress mitochondrial respiration.

However, not all tissue respiration follows this pattern. Germination of *Cucumis sativa* (cucumber) and *Vigna radiata L. (mung bean)* seeds show a "break" at 13°C indicating the process is chilling sensitive whereas the rate of respiration of the imbiled seeds decreased in a linear fashion as the temperature was reduced to 5°C (Simon *et al*, 1976). Similarily linear Arrhenius plots have been obtained for pollen respiration of tomatoes (Patterson *et al*, 1979). Mitochondrial respiration of *Cornus stolonifera* callus shows chilling injury below 13°C though intact plants of this species are frost tolerant (Yoshida and Tagawa 1979). These results suggest that although the mitochondria of chilling sensitive plants respond more dramatically to lower temperature than those from tolerant plants under the same circumstances, disruptions of mitochondrial respiration might not be the primary effect.

In some species, however, mitochondrial cold sensitivity may be sufficient to account for sensitivity of the plant. For example, *Episica reptans*, a tropical lowland plant which is extremely cold-sensitive, shows dramatic increase in respiration within 2 hours of chilling (Wilson 1979). This increased respiration rate is also seen in *Xanthium strumarium* (Drake and Raschke, 1974). This observation of massive oxidative reactions has tentatively been attributed to peroxidation of disintegrating membrane components and general permability of cell membranes (Wilson 1979). These observations led the author to propose two categories of cold sensitive plants. The first class is extremely chilling sensitive and cannot be chill hardened at 12°C and 85% relative humidity (R.H.), or drought hardened at 25°C and 40% R.H. Even prolonged periods of acclimation at 15°C results in little increase in chilling tolerance. Maintaining a saturated atmosphere at 5°C does not delay the onset of injury (eg,*E. reptans, E. cupreata, N. lynchii*). The second class is less sensitive to chilling and drought and can be protected against chilling injury at 5°C for 9 days if the atmosphere is water saturated. Also these plants can be chill- and drought-hardened to some degree (eg *Phaseolus vulgaris, Cucumis sativus, Gossypium hirsutum*) (Wilson 1979).

Obviously these categories are based on artifical distinctions. In nature one would expect to find a continuous gradation of chilling sensitivity such as that found in related *Passiflora species.* The Lyon - Raison hypothesis is flawed for a similar reason since it predicts a critical temperature below which some vital process cannot be maintained. However, plant survival is dependent not only on temperature but also irradiance, (McWilliam and Naylan 1967) humidity , (Wright and Simion 1973) and the amount of cold hardening it has received (i.e.,exposure to low but not chilling temperature) (Guinn 1971). Thus, there is no critical temerature below which a plant always dies and above which it always lives. Furthermore the temperature at which a break occur in the slope of an Arrhenius plot depends on the parameter been measured. For example, the rate of increase in dry weight , leaf area, and length of hypocotyl plus radicle of *Vigna radiata* L. (mung bean) and *Pharbitis nil* chois (morning glory) all show marked responses to decreased temperature, although when drawn as Arrhenius plots the responses resembles a smooth curve rather than a set of intersecting lines (Bagnall and Wolfe 1978). This has led the authors to argue that changes in the membrane fluidity might not be expected to cause abrupt changes in the slope of Arrhenius plots at either the enzyme or whole plant level. A bilayer made of one species of lipid only and containing no protein, will, over a small range of temperature, change its molecular mobility drastically from fluid to solid state. The bilayer in the membrane of a living organism is more complex (Lyon *et al*, 1964). Several or many species of lipids are present and proteins are embedded in the membrane. As the temperature of such a mixture is lowered it separates into domains of different composition and thus small regions of frozen lipid appear in an otherwise fluid bilayer (Lee 1977). These increase in size as the temperature is lowered until eventually the whole bilayer is frozen. Therefore the bilayer is composed of domains of various compositions some frozen, some fluid over a wide temperature range. Proteins further complicate matters by perturbing the membrane. The boundary layer of lipids around a protein is in a different environment from those of bulk lipids (Jost *et al*, 1973). Thus, it may be argued that changes in conformation will not occur in all membrane proteins at the same temperature due to the complex composition of the membrane. The implication is that enzyme activity is a continuous function of temperature. Perhaps this might explain, in part, the inconsistency of Arrhenius plots in determining phase transitions.

Photosynthesis

One common feature associated with chilling injury is a reduction in photosynthesis which occurs 12 - 24 hours after chilling (Berry and Bjorkmann 1980; Martin *et al*, 1981). This observation is generally consistent with the Lyon-Raison hypothesis of phase transitions since many of the reactions of the photosynthetic process are associated with membranes. Temperature induced changes in the bilayer structure or composition of these membranes might be expected to have significant effects on the overall rate of photosynthesis.

In general, reduction of photosynthetic capacity is progressive with increased time of exposure to low temperature. This has been interpreted as evidence that the maintainance of the photosynthetic apparatus is affected rather than inactivation of a specific component of the photosynthetic apparatus.

Chlorosis is apparent in many thermophillic plants subjected to chilling temperature (Slack *et al*, 1974; McWilliam and Naylor 1967). However in *Lycospersicon* (tomatoes) and *Xanthium strumarium* L. a thermophillic weed, there is little decrease in the chlorophyll content of the leaves as photosynthesis declines (Drake and Raschke 1974; Martin *et al* 1981). Furthermore, this decrease in chlorophyll cannot account for the overall drop in photosynthesis.

Prechilling also causes a reduction in stomatal aperture (Drake and Raschke 1974) and has led to the proposal that this effect might play a role in reduction of photosynthesis by causing increased stomatal resistance to

CO_2 flux (Crookstan *et al*, 1974). The results of studies in which net photosynthesis is measured at different CO_2 concentrations in the ambient air conflict with this hypothesis (Drake and Raschke 1974; Martin *et al*, 1981). The reasoning behind their experiments was that stomatal contributions at normal atmospheric CO_2 concentrations can be overcome if any decrease in intercellular CO_2 concentrations are reversed by increasing the ambient CO_2 levels. Any remaining inhibition of photosynthesis after readjustment of intercellular CO_2 concentration to the level existing prior to chilling must be due to direct impairment of one or more processes in the chloroplast. Results of experments with both tomatoes and *Xanthium* show photosynthesis remained depressed by up to 37% in chilled leaves versus controls. Furthermore the intercellular concentration at which one half the maximum rate of net photosynthesis was attained was the same in prechilled and control plants. This suggests that prechilling affects the maximal rate of net photosynthesis but not the apparent affinity of the assimilatory apparatus for CO_2 .

Starch accumulates in the cold in leaves of some species and can damage the photosynthetic apparatus (Forde *et al*, 1975) . However in *Sorghum bicolor* starch levels depend on the stage of diurnal period that chilling is applied. Less starch accumulates in *Pennisetium* cultivars grown at 18°/13°C compared to 24°/19°C and starch grains visibly decrease in size on transfer from high to lower growth temperatures (Pearson *et al*, 1977)

When leaves of *Sorghum bicolor* were fed $^{14}CO_2$ at 10°C in the light, label accumulated in aspartate and was slow to move into normal photosynthetic pathways (Brooking and Taylor 1973). Several cold tolerant or partially cold tolerant species (*Lolium sp.*, *Paspolum dilatatium*, and *Glycine max*) showed a large accumulation of free amino acids related to C4 metabolism suggesting a disturbance in normal C4 photosynthesis. The photosynthetic PEP carboxylase of some C4 plants is cold sensitive (Uedan *et al*, 1976) and could account at least partially for depressed photosynthesis in C4 species in the cold.

Pyruvate othophosphate dikinase, an enzyme which serves in C4 photosynthesis to regenerate the primary CO_2 acceptor dissociated to an inactive form at low temperature and the rate of inactivation *in vitro* differs according to the species from which the enzyme is extracted (Shirahashi *et al*, 1978; Sugiyama *et al*, 1979). The enzyme from species that are successful at low temperature is more stable at low temperature. Effects of cold treatment *in vivo* on the extractable enzyme activity of pyruvate othophosphate dikinase seems to be correlated with the overall sensitivity to the cold of the species used (Sugiyama *et al*, 1979).

Many plants with the C4 pathway of photosynthesis are chilling sensitive and at one time it seemed likely that some step in C4 metabolism was unusually sensitive to low temperature. As noted in the previous discussion no one step can be found. Recently two C4 species native to cool regions were found to grow well in laboratory studies at 8 °C and biochemical studies did not detect any disruption of the normal metabolic sequence of C4 photosynthesis at low temperature (Caldwell *et al*, 1977). Many C3 species from these regions are also chilling sensitive. Thus, the sensitivity of these C4 plants to chilling is probably not characteristic of C4 photosynthesis.

The effect of temperature on photochemical activities has been compared in thylakoids isolated from chilling sensitive and chilling resistant plants (Margulies 1972; Shenyour *et al*, 1973). *Phaseolus vulgaris* (bean) leaves that have been aged for less than 2 days at 0°C yield thylakoids with less than 5% DCIP or $NADP^+$ Hill reaction activity as compared to fresh leaves. This activity is rapidly regained when leaves are placed in the light (Margulies 1972). Although photosystem II appears to be affected, photosystem I activity is not lost since, when thylakoids from aged leaves are supplied with DCIP and ascorbate, they reduce NADP.

Many of these original results are now being questioned because of the finding that both darkness and detachment of leaves from the plant are more effective than cold treatment in damaging photosystem II (Kanuiga *et al*, 1978). When tomato plants are exposed to 0°C in intermittant light, Hill reaction activity is not affected while dark storage at either 0°C or 25°C results in significant decrease in Hill reaction activity after 2 - 3 days. Thylakoids prepared from two chilling sensitive plants, bean and tomato, and two chilling resistant plants pea and lettuce, were measured for photochemical activity over a temperature range of 3 - 27°C. Both the photoreduction of $NADP^+$ from reduced DCIP and ferredoxin $NADP^+$ reductase (EC1.6.99.4) activity of chloroplasts of chilling sensitive plants show increase in activation energies (Ea) at approximately 12°C whereas photosystem II activity of the thylakoids shows a constant activation energy over the temperature range. The chilling resistant plants showed constant activation energies for $NADP^+$ photoreduction over the same temparature range. The results suggest that chilling effects are localized to photosystem I (Shneyour *et al*, 1973). Others have found that thylakoids isolated from *Spinacia oleracea* L. (spinach) a chilling resistant plant were inactivated when exposed to light at 4 °C. This observation creates uncertainty as to whether resistance of spinach thylakoids *in vivo* to chilling temperature is solely a function of the chloroplast thylakoid membrane and suggests caution when using *in vitro* results to make inferences about *in vivo* observations. The studies on photosynthesis described here demonstrate that the process can be broken down into 3 categories: biochemical, CO_2 diffusion, and photochemical. Each of these can be affected differently by diverse factors present in the surrounding environment. Since any of these three processes can be limiting under different environments, plants have most likely evolved mechanisms of adapation to overcome a particular limitation. Temperature could effect these three processes of photosynthesis in different ways. For example disruption in membrane function should effect photochemical reactions since they are embedded in membranes. Dark reaction enzymes are soluble and should not be affected directly by membrane disruption. The diffusion of CO_2 from the atmosphere to the site of carboxylation is affected by temperature, since diffusion is a temperature dependant parameter. This in turn would affect biochemical reactions of the Carbon cycle which are also dependant on temperature and on CO_2 concentration at the site of carboxylation. The point is that each one of these processes can be directly affected by temperature which will in turn affect the other processes since they depend on one another. This makes physiological studies on the primary effects of temperature on photosynthesis very difficult to interpret. A systematic study of a simple system or mechanism will most likely involve direct measurment of its components and factors that directly affect the system. However, for more complex systems such as chilling effects and mechanisms of chilling tolerance this type of study has failed and a different approach should be taken. This has led to the approach of using genetically controlled differences for studies of temperature effects on plant systems.

Genetic Studies

Little is known at present about regulation of gene expression for chilling sensitivity by biochemical and environmental factors. Of obvious interest is the role of temperature itself as a potential regulator of gene expression. Of the genetic studies done in determining the mechanisms of chilling sensitivity or resistance three major approaches have been used. The first method is to study species which are closely related and yet which have very different degrees of chilling resistance. In this way it is hoped that genotypic factors not connected with adaptation to different temperatures would be minimized. For example, studies with *Passiflora* species with varying tolerance to temperature stress have shown that species originating from tropical lowlands are much more susceptible to low temperature damage than those from higher altitudes and cooler environments (Patterson *et al*, 1976). Using electrolyte leakage as an indicator of resistance, *Passiflora* species were ranked for sensitivity to chilling temperatures. Plants that leaked electrolytes after a 6 hour period were classified as chilling sensitive. The amount of leakage determined the the degree of sensitivity. Using this method, they classified the species in the order *P.caerulea* < *edilis* < *cincinnata* < *maliformis* < *flavica rpa.* F_1 hybrids such as *P. flavicapra* x *cincinnata* occupied intermediate positions , although the F_2 individuals of *P. maliformis x caerulea* and *P. flavicarpa x caerulea* rank with *P. caerulea* in this test. There is a continuum between the sensitive and resistant ends of the range suggests that chilling resistance in *Passiflora* is not an all or none response, but depends on some kind of gene dosage effect. The expression of a number of genes in an additive way could explain the results.

The second method used to study gene expression of chilling resistant has involved the formation of somatic hybrids of chilling sensitive tomatoes and chilling resistance potatoes (Melchers *et al*, 1977). Somatic hybrids are formed by the fusion of two phenotypically marked protoplasts from different species. Susceptability to chilling injury, as indicated by changes in the dark reduction of cytochrome f in leaves stored at 0 °C was measured in four of the somatic hybrid tomato-potato hybrids. All four showed enhanced resistance compared with tomato, but were less resistant than potato. As before, this can be interpreted as evidence that there are multiple genes involved in chilling resistance.

However a certain amount of caution should be employed in interpreting the results obtained from the two previous methods. All plants used in all these experiments are seperate species and have been grown in quite different environment Plants evolve to adapt to a complete range of environmental variables, if any of those environmental components extend beyond that range the plant will be stressed. For example, *Passiflora* species native to high altitude environments have not only adapted to chilling temperatures but must also adapt to less humidity and different CO_2 and O_2 levels than species growing at sea level. Therefore adaption to a certain environment is a complex phenomonen and not necessarily restricted to one factor in that environment.

The third general method used in understanding the genetic mechanisms of chilling resistance has involved plant tissue and cell culture. The isolation of chilling resistant cell lines from *Nicotiana sylvestris* and *Capsicum anniuum* callus culture has been reported (Dix and Street 1976). These lines were obtained by selecting for survival of ethyl methane sulfonate (EMS) treated cells submitted to chilling for 21 days at -3°C and +5°C respectively. Unfortunatly tobacco plants could not be regenerated from some cell lines and callus derived from seedling progeny was sensitive in others. (Dix 1977). Plants could not be regenerated from resistant or sensitive *C. annuum* cultures. It was concluded that the loss of resistance in *N. sylvestris* resulted from a fairly stable epigenetic change, persistent through an indefinite number of mitotic divisions in cell culture, but lost during the plants sexual cycle. Alternative explanations for the loss of resistance, such as continual segregation in culture giving rise to chimeral plants cannot be ruled out.

In principle, the problems encountered in interpreting the effects of low temperature on hybrids of genetically distinct plants or callus tissue culture could be circumvented if it were possible to compare plants which are genetically identical except for single mutations which alter their cold sensitivity. This approach would offer the distinct advantage of genetic analysis of each component that confers hardiness.

There are some conceptual problems with this approach. If a mutation is introduced into a cold hardy plant which makes an enzyme cold temperature sensitive, (i.e., a missense mutation) and this enzyme is vital to the plants viability it would render the cold hardy plant cold sensitive. However, the mechanism of cold sensitivity would have nothing in common with that seen in natural systems. Conditional cold sensitive mutants have been isolated in bacteria and lower eukaryotes (Russel *et al*, 1980; Picard-Bennoun and Le Cage 1980). The majority of these are defective in the biosynthesis of cytoplasmic ribosomes, although some bacterial cold sensitives are auxotrophs (Abd-el-al and Ingraham 1969). It is probably possible to make many enzymatic functions cold - temperature sensitive. That such mutations have not been observed more frequently may simply reflect that the effort has not been as intensive as for the identification of hot - temperature sensitive mutants.

One possible way to distinguish between a mutation disrupting a chilling resistance function in an organism and a simple temperature sensitive (Ts) mutant is that the former should always be expressed but its effects will not be seen under non permissive conditions. A normal cold Ts mutant will only show altered metabolism under restrictive temperatures. An exception to this criterion would be if the natural mechanism of chilling resistance is inducible, in which case the mutation confering temperature sensitivity would not be expressed in all conditions. The possibility that an inducible resistance system exists can be tested. The wild type cold hardy plant should show changes in some aspect of metabolism as the temperature is lowered. In contrast the chilling sensitive mutant should show a different pattern of metabolism with respect to wild type as the temperature is lowered because the resistance mechanism(s) cannot be induced. Such analysis might, however, become complicated because of dramatic changes in many aspects of metabolism as the sensitive mutant becomes stressed.

It should be emphasized that many functions in the mutant should show alterations under non permissive conditions if the mechanism for resistance is central to all stages of development, tissue and/or organelles of the plant. Thus if sensitivity is dependent on the tissue/organ exposed or the stage of development this implies the plant has a number of mechanisms for coping with chilling temperatures or that the structures affected by chilling are not present in all areas at different stages of the plant life cycle. If one were to find a plant system which only showed sensitivity to the cold at certain stages of its development this might provide clues as to what mechanisms are involved in chilling resistance.

In principle, the isolation of mutants with defects in "cold tolerance genes" should allow one to minimize both genotypic and environmental variables thereby facilitating physiological analyses of chilling sensitivity. If mutants with defects in the mechanism(s) involved in chilling resistance can be isolated it should permit delineation of the complexity of responsible mechanisms by complementation studies and investigation of whether a second site mutation can restore resistance to the plant. Such information could be instructive in attempts to make chilling sensitive plants resistant.

Arabidopsis thaliana (L.) Heynh is well suited for such a study as it is normally a cold hardy plant. In addition, the small size and short life cycle facilitates screening of large populations of M2 plants for cold sensitive mutants (Redi 1975).

This study describes the preliminary analysis of one such variant which exhibits extreme chilling sensitivity and dies after four days at temperatures below 16°C but is essentially indistinguishable from wild type at temperatures above 22°C. It is believed to be the first report of a mutation which confers cold sensitivity on a higher plant. Preliminary evidence suggests the cold sensitive mutant has altered lipid metabolism.

II. MATERIAL AND METHODS

Growth Conditions

Arabidopsis thaliana (L.) Heynh., race Columbia, was used in this study. The seed source for the mutant search was an M2 population of ethyl-methane sulfonate treated seed provided by C.R.S. (Somerville and Ogren, 1979). Mutant PM11 was originally retained in a screen of M2 seedlings at the 4 leaf stage for plants sensitive to high levels of CO_2 (1%) but was subsequently found to be cold temperature sensitive.

Physiological experiments were preformed on M4 or M5 advanced generations. All plants, wild type and mutant derivatives were grown under continuous flourescent illumination (200 - 300 uEinsteins $m^{-2}s^{-1}$) at 21°C and 60% relative humidity. Most physiological and phenotyping experiments were done under the same conditions except the temperature was 13°C.

Plants were sown by suspending seeds in a 0.15% agar solution and pipeting approximately 100 seeds into 5 inch pots containing a perlite:vermiculite:sphagnum mixture (1:1:1) overlaid with fine vermiculite. Pots were soaked with a nutrient solution (1 litre/pot) before seeding and wrapped in saran wrap for one week. Once seedlings were established they were watered twice a week with nutrient solution and every other day with water. The nutrient solution is prepared from the following stock solution:

1.0 M KNO_3, 5 ml

1.0 M KPO_4 (adjusted to pH 6.50), 2.5 ml

1.0 M $MgSO_4$, 2.0 ml

1.0 M $Ca(NO_3)_2$, 2.0 ml

1.8% (w/v) Sequstrene 330 Fe, 2.0 ml

Micronutrient solution (70 mM H_3BO_3, 14 mM $MnCl_2$, 0.5 mM $CuSO_4$, 1 mM $ZnSO_4$, 0.2 mM $NaMoO_4$, 10 mM NaCl, 0.01 $CaCl_2$), 1 ml

H_2O 985.5 ml

For certain experiments, plants were grown under sterile conditions on petri plates containing a 2% agar medium with the same chemical composition as the nutrient solution. Seeds were surface sterilized by soaking in a Tween-80 (2 drops/100 ml), 10% chlorox solution for five minutes. This was followed by three washes in 70% ethanol and three washes in distilled water. The petri plates were sealed with parafilm. Plants could be grown to reproductive maturity under these conditions.

Genetic Crosses

Arabidopsis is normally a self pollinating species. Mutant and wild type plants were crossed by emasculating a mutant female flower and hand pollinating with the wild type used as the male. F_1 plants from these crosses were tested for the cold temperature phenotype. The phenotype was scored by a yellowing of plant leaves after a four day exposure to non-permissive temperatures (13°C). F_2 seed was collected and tested for segregation studies of the mutant phenotype.

Gas Exchange

Photosynthetic CO_2 - fixation and dark respiration was measured by a system similar to that described by Somerville and Ogren (1982). An Analytical Development Co. Infrared Gas analyser (IRGA) type-225 was used to measure CO_2 concentrations in the system. Dry gas of desired composition was humidified to about 70% to avoid descication of the plant. The gas was then passed through a chamber, containing a single plant, which was immersed in a circulating water bath so that the temperature at which gas exchange occured could be controlled. The gas stream was then dehumidified before entry to the IRGA by passing through a cold finger at 4°C and CO_2 levels in the gas stream were measured in the differential mode. The flow rates for this open system were determined by a Tylan mass flow meter. Light intensity was approximately 350 uEinsteins $m^{-2}s^{-1}$. The gas composition, flow rate and temperature are given with each experiment.

$^{14}CO_2$ Labeling and Separation of Products

Short term $^{14}CO_2$ labeling of products of photosynthesis was done as described by Somerville and Ogren (1982). Four plants were equiliberated in the dark for about 10 minutes, the system was then closed and 10 uCi of carrier free $NaH^{14}CO_3$ was injected into a resevoir of 8% phosphoric acid. After a 15 minute labeling period plants were removed and killed by immersion in liquid N_2. Plants were transferred to a TenBroek homogenizer and ground in 80% ethanol. Insoluble matter was removed by centrifugation (5 min at 5000Xg), dried, and resuspended in 350 ul of perchloric acid; 30% H_2O_2: water solution (3:2:2)., The solution was heated for a few hours to 100°C, cooled scintilation fluid was added and the samples were counted in a scintilation counter.

The chlorophyll (chl) concentration was determined by measuring the absorbance of the supernatant at 654 nanometers and transforming according to the following formula: (Winterman and Demots, 1965)

$(A_{654} * 39.8)\ 1000 = \text{mg chl/ml}$

This supernatent was dried and resuspended in water for further analysis.

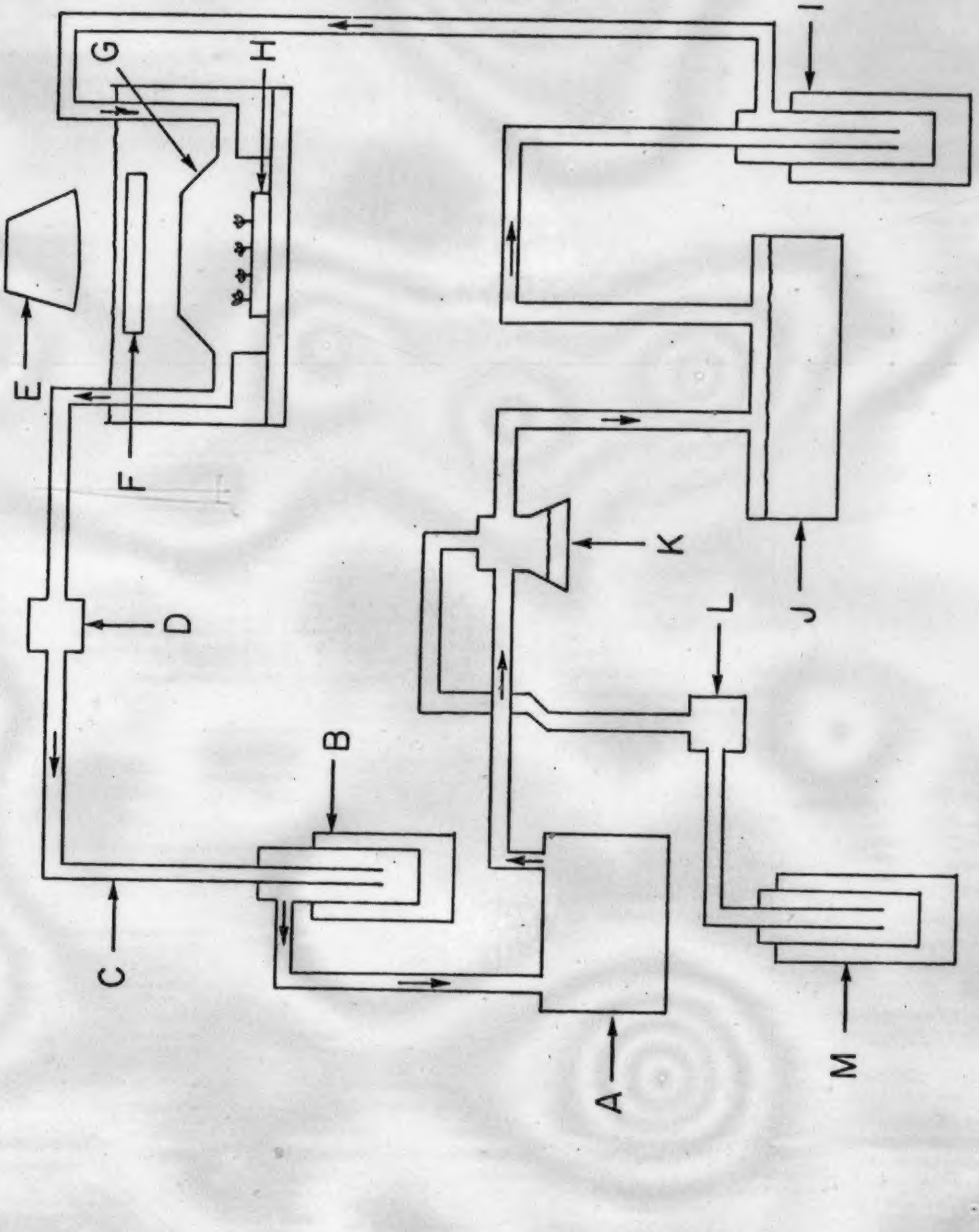
Labelling for longer periods (eg. 24 hours) was done on 8 to 14 plants simultaneously using a system diagramed in Figure 1. This closed system is similar to the one previously described except a gas analyser is connected in line. A solution of $NaH^{14}CO_3$ (9.5 uCi/umol) was introduced into the system at a constant rate using a varioplex II pump (LKB 2120 model). Balanced between the CO_2 evolved by acidification of bicarbonate and CO_2 fixation by the plant was monitored by the gas analyser and regulated by adjustment of the flow rate of bicarbonate into the acid flask. The total size of the system was approximately 700 ml. The circulation rate of gas was 500 ml min^{-1}, humidity was 70%, and light conditions were 350 uEinsteins $m^{-2}s^{-1}$. Specific conditions are given in the text for each experiment.

Figure 1

Schematic diagram of the long term labeling apparatus

(A)CO_2 gas analyser; (B)Ice bath dehumidifier; (C)Tygon tubing; (D)Gas pump; (E)Light source(300W Incadesent) ; (F)Infrared filter(G)Plant chamber (H)Plant holder; (I)21°C water bath dehumidifier; (K) 8% phosphoric acid flask; (L)Peristaltic pump; (M)Bicarbonate solution ice bath.

Figure 1


A
B
C
D
E
F
G
H
I
J
K
L
M

Labelling was terminated as previously described except plants were ground in a chloroform:methanol:water (5:12:1) solution. This homogenate was washed twice with a 0.9% NaCl solution. The washes were combined and consisted largely of polar labeled components such as amino acids, organic acids, and sugar phosphates. The chloroform phase contains all non-polar ^{14}C labled compounds ~~such~~ as pigments and lipids.

Analysis of products of $^{14}CO_2$ photoassimilation from the soluble aqueous fraction was achieved by applications of 2 ml aliquots to a Dowex-50 (H^+) column (0.5 x 3.5 cm). The column was washed with 2.5 ml H_2O and the wash was saved for further separation of neutral and acid components. The compounds binding the Dowex-50 (H^+) column was eluted with 8 ml of 2N NH_4OH solution. This eluate is designated as the basic fraction and is predominently amino acids.

The neutral and acid wash from the Dowex 50 (H^+) column was applied to a Dowex-1 (formate) (0.5 x 2.5cm) column and washed with 2.5 ml of H_2O. The wash contains the neutral fraction which is largely sucrose. The Dowex-1 column was then washed with 8 ml of 0.5 N formic acid followed by 8 ml of 8 N formic and finally 6 ml of 4N HCl. The first or Acid-1 fraction contains primarily organic acids, the second fraction or Acid- 2 fraction consists mostly of sugar monophosphates. The Acid-3 or third fraction is largely sugar diphosphates. Seven hundred ul aliquots were counted in scintillation fluid and percent distribution of ^{14}C assimilate was determined for each fraction.

In the case of long term labelling, the acid fraction was not subfractionated but removed as one fraction with 6 ml of 6N HCl.

The fractions isolated from ion exchange columns were further separated by thin layer chromotography (TLC). Fractions were dried under vacuum to concentrate the samples. Samples were resuspended in water and aliquots containing 20,000 counts per minute were spotted on TLC cellulose plates in a volume of about 10 ul. (Analteck Cellulose MN 3000 250 micron). All plates were equilibriated in chromotography tanks for about 45 minutes before development.

Compounds in the basic fraction were separated using n - butanol:acetone:water:diethylamine (20:20:10:3) solvent Cossins and Sinha, 1966). A solvent containing n-butanol: acetic acid: H_2O (3:1:1) (Brenner and Niederwieser, 1960) was used if a second dimension was needed. Standards were detected with a ninhydrin reagent (0.1% ninhydrin in aqueous butyl alchol).

Organic acids from the Acid- 1 fraction were separated using diethyl ether:acetic acid:H_2O (13:5:1). Standards were detected by spraying with bromophenol blue reagent (0.04% Bromophenol blue in 95% ethanol pH 6.5) (Buch, 1965).

The acid fraction from long term labelling experiments was separated using 2 dimensionsal systems. The first dimension was EDTA: 17N NH_3OH H_2O: n-propanol: isopropanol: n-butanol: isobutyric acid (1.2g:100:950:350:75:75:2500) solvent (Crawley *et al*,, 1963). The second solvent used was a mixture of equal volumes of proponoic acid:water (180:220) and butanol:water (370:25) solvent (Benson *et al*, 1950).

For separations of the non-polar fraction, samples were dried under N_2 and resuspended in chloroform. Samples were spotted and plates developed as soon as possible to minimize oxidation of liquid compounds. Silica thin layer chromotography plates were used (Baker Si 250 P.A. 200 microns). Plates were activated by heating to 100°C for 30 minutes, cooled and then spotted with 10 - 20 ul of sample.

For the neutral compounds the following solvent system was used: petroleum ether (B.P. 60 - 80°C): diethyl ether:acetic acid (75:25:1). The system for the phospholipids was a two dimensional system (Nichols and James, 1966). The first dimension was chloroform:methanol: 7N ammonia (65:25:4), and the second dimension was chloroform: methanol:acetic acid:water (170:25:24:4). Standards were detected by exposing plates to iodine vapours for approximately one hour.

All plates were dried, sprayed with autoradiograph enhancer (NEN Enhance), and allowed to autoradiograph for 2 - 4 days at -70°C. Compounds were visualized on X-ray film (Kodak X-OMAT AR). Quantification of label was done by removing the area corresponding to a spot on the X-ray film, digesting the sample in a solution of perchloric acid:H_2O_2:water (3:2:2) and heating to 100°C for a few hours. Scintillation fluid was added and the sample counted.

Photosynthetic Electron Transport

Isolation of broken chloroplasts was carried out under dim light. Four hundred mg of prechilled leaves from both wild type and PM11 were homogenized in 15 ml of grinding buffer (50 mM Tricine (pH 7.8) 10 mM NaCl, 10 mM EDTA, 400 mM sorbitol) using three 5 second pulses at the top speed of a VerTis homogenizer. The homogenate was passed sequentially through cheese cloth and miracloth before centrifugation at 5,000 g for 4 minutes. The supernatant was discarded, tubes were dried and the pellet was resuspended in 1 ml of washing buffer (10 mM Tricine (pH 7.8), 10 mM NaCl, 5 mM EDTA) to ensure lysis of chloroplasts. The suspension was centrifuged as before, the supernatant was discarded and the pellet was resuspended in 200 ul. of resuspension buffer (500 mM Tricine (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, 100 mM sorbitol). The chlorophyll concentration was determined from an aliquot of this sample.(Winterman and Demots, 1965) Thylakoids were stored on ice until use.

Electron transport was measured in a Rank O_2 electrode at 23°C at a light intensity of 1,000 eisteins $m^{-2}s^{-1}$ at the surface of the cuvette. The final volume was usually 2 ml Thylakoids (20 ug chlorophyll) were added to 1.9 ml of resuspension buffer containing 1mM Gramicidin-D as an electron transport uncoupler. The complete reaction was assayed by using methyl viologen as a terminal electron acceptor (Izawa 1980). Photosystem II activity was measured by the rate of O_2 evolution with ferricyanide (Fe^{3+}) as an electron acceptor. Photosystem I activity was measured following diferential inhibition of Photosystem II by the herbicide DCMU. Ascorbate in conjunction with DCIP were used to supply electrons directly to Photosystem I and removal of O_2 via methyl viologen was used as an indicator or electron transport rates. (Izawa 1980).

Gas Chromatography

Samples were prepared by grinding leaves in chloroform methanol (1:1) solution followed by drying under N_2 (Folch *et al*, 1957). Fatty acid methyl esters were prepared by tranesterification in the presence of 5% HCl in absolute methanol 80°C in sealed vials . GLC analysis was carried out using a Varion model 3700 instrument equipped with a flame ionization detector. A 37.5 meter silica column (Carborwax 20 m column) with N_2 as carrier gas was used. Operating temperatures for the column, injection port, and detector were 200°C, 230°C and 240°C respectively. Quantification was done by a Hewlett Packard Auto lab integrator. Peaks were identified by comparing the times they were detected by the flame ionizer with a known standard mixture of fatty acids.

^{14}C - Acetate Labelling

The procedure used for labelling *in vivo* is similar to that described for spinach leaves (Slack and Roughan, 1975). Twenty ul of 0.5 mM sodium (1-^{14}C) acetate (56 m Ci/m mole) in 80% ethanol was applied to the surface of expanding *Arabidopsis* leaves. The droplets evaporated in about 10 minutes and plants were left at 22°C for 12 hours. Plants were washed thoroughly to remove unabsorbed acetate from the leaves. A sample of the leaves was taken for separation of ^{14}C acetate-labelled products and plants were shifted to 13°C where samples were taken 12, 24 and 48 hours after transfer.

Labelling was stopped by washing the leaves in water and grinding in chloroform:methanol:water (5:12:1). Separation was done as described earlier for the long term $^{14}CO_2$ labelling experiment (24 hours). The non-polar phase was dried and chromatographed using the system described for phospholipids.

Spots were quantified by dampening the area to be removed with water then scraping the silica into a 5 ml scintillation vial. Three hundred and fifty ul of chloroform:methanol (2:1) was added to elute the nonpolar compounds. Sintilation fluid was added and the sample was counted.

III. RESULTS

Mutant Isolation

The *Arabidopsis* mutant line PM11 was originally isolated from a population of approximately 10,000 M2 (EMS - mutagenized) plants (Somerville and Ogen, 1979) on the basis of an apparent sensitivity to high levels (1%) of atmospheric CO_2. It was subsequently discovered that the line was not sensitive to CO_2 but was extremely chilling - sensitive. The mutant exhibits normal growth at temperatures above 22°C but turns yellow and dies when exposed to temperatures below 16°C. The older leaves become chlorotic before the younger ones and after 6 days of chilling no green tissue remains.

The presence of the mutation is scored by shifting plants to 13°C for two days at which time the mutant phenotype is distinguished. The plants are then rescued by returning them to 22°C. As this generally leads to substantial damage to the leaves, such plants were not used for physiological experiments but were used as a source of seed.

The original M3 mutant line segregated a small white seedling which did not produce true leaves and died early in development. The mutation responsible for this phenotype was separated from the mutation confering cold senistivity by three generations of single seed selection.

The effects of the mutation on growth are apparent from the results of the experiment presented in Plate I.

Plate I

Effect of temperature on growth of wild type and mutant PM 11.

Seedlings were grown on sterile nutrient medium to the rosette stage at 22°C then: (A) shifted to 13° C for 5 days, or (B) maintained continuously at 22°C.

A

B

Plate 1

PM11
WT
PM11
WT

experiment seeds were germinated in petri plates under sterile conditions at 22°C and grown to the four leaf stage. Petri plates were used so as to keep the environment in which the plants were growing as uniform as possible. One plate was shifted to 13°C for 4 days and the other was maintained at 22°C. There is no apparent difference in the growth of the mutant and wild type at 22°C (Plate Ib), but at 13°C the mutant had lost colour and stopped growth (Plate Ia).

Genetic Studies

The chilling sensitive phenotype of PM11 was carried though the M3 and subsequent generations suggesting a genetic basis to the effect. F_1 plants from a PM11 x WT cross, exposed to 13°C for 5 days, did not show the cold sensitivity effect. However, F_2 plants from the cross segregated 3:1 (294 green plants, 87 yellow plants; $x^2=.874$ $p > 0.3$) implying that a single nuclear recessive mutation is responsible for the cold sensitivity. The lack of mutant phenotype in the F_1hybrid was consistent with this hypothesis. The mutation responsible for the effect was designated *colD1*.

The yellow F_2 segregants from the PM11 x WT cross were shifted back to 22°C after 3 days of exposure to 13°C and plants began to recover demonstrating that the mutation was still a conditional lethal. Plants were able to produce seed although not as much as the unstressed mutant. This seed was recovered and will be used to advance subsequent lines of the *colD1* mutant.

Effect of Developmental Stage

The developmental time of expression of the *colD1* mutation was studied to determine if the defect was expressed at all stages of development. If PM11 showed a specific developmental stage which it was chilling resistant this would suggest that the *colD1* gene is developmentally regulated and not needed at all stages of development, or that other genes of similar functions are expressed at various times in the development of *Arabidopsis*. The method of testing whether the *colD1* mutation was stage specific was to study seed germination under sterile conditions in petri plates in chilling conditions (13°C). Germination was measured by appearance of cotyledons. Both wild type and PM11 required approximately 10 days at 13°C for 95% germination. Of 150 seeds of PM11 placed under these conditions all but a few germinated and produced green cotyledons similar to wild type. However, when the true leaves began to appear, a yellowing of the centre of the plant became obvious. The true leaves appeared yellow and eventually the plant turned completely yellow and died at a very early stage.

This observation suggests that the effects of the *colD1* mutation are stage specific and do not effect germination or cotyledon metabolism. It is only when the true leaves and seedling growth start that the effects of chilling are seen. Either the *colD1* mutation is not expressed in the seed or cotyledons, or another gene of similar function to the *colD1* gene is expressed in the seed and cotyledon.

Onset of Injury Following A Temperature Shift

The experiments described here were designed to determine the rate or time of onset of injury caused by prolonged exposure of PM11 to chilling temperature, and to determine at what point injury was irreversible. Chlorosis was used as a general indicator of injury as it is a common response of plants to stress and is relatively easy to quantify. Chlorosis may occur because of depressed synthesis of chlorophyll precursors or because the rate of turnover or photodestruction of chorophyll and associated pigments occurs more rapidly than synthesis.

A population of genetically uniform plants grown in soil in pots frequently show heterogenity in growth and development due to microenvironmental differences in the soil. In order to alleviate concern about environmental variation sterile seeds of mutant and wild type were placed in minimal nutrient medium in petri plates. One plate was incubated at 13°C for various times then returned to 22°C in order to determine if the the mutant showed recovery. The six treatments were:

a) Six days at 22°C.
b) One day at 13°C, 5 days at 22°C.
c) Two days at 13°C, 3 days at 22°C.
d) Three days at 13°C, 3 days at 22°C.
e) Four days at 13°C, 2 days at 22°C.

f) Six days at 13°C.

At one day intervals, two plants of mutant and wild type were removed from each treatment, fresh weight was determined and plants were ground in 95% ethanol to determine chlorophyll. The results of this experiment are presented in Figure 2.

The chlorophyll content of both PM11 and wild type remained constant and fairly similar when measured over a six day period at permissive temperatures (Fig. 2a). In contrast, the effects of the *colD1* mutation are readily apparent as a dramatic loss of chlorophyll content in plants maintained at 13°C for six days (Fig. 2f).

There appeared to be only a marginal decrease in chlorophyll levels in PM11 versus wild type up to two days of exposure to chilling temperatures. However, plants exposed to longer periods of cold temperatures or shifted to permissive temperatures after 2 days showed marked chlorsis (Fig. 2c, d, e, f). Figure 2b and 2c demonstrate that the mutant does not have to be constantly subjected to chilling temperatures in order to induce chlorosis. Plants removed after 2 days still appear healthy but after one day at 22°C they show a decrease in chlorophyll. This 24 hour lag in response is also seen in the recovery of PM11 after a shift back to 22° C (Fig. 2d).

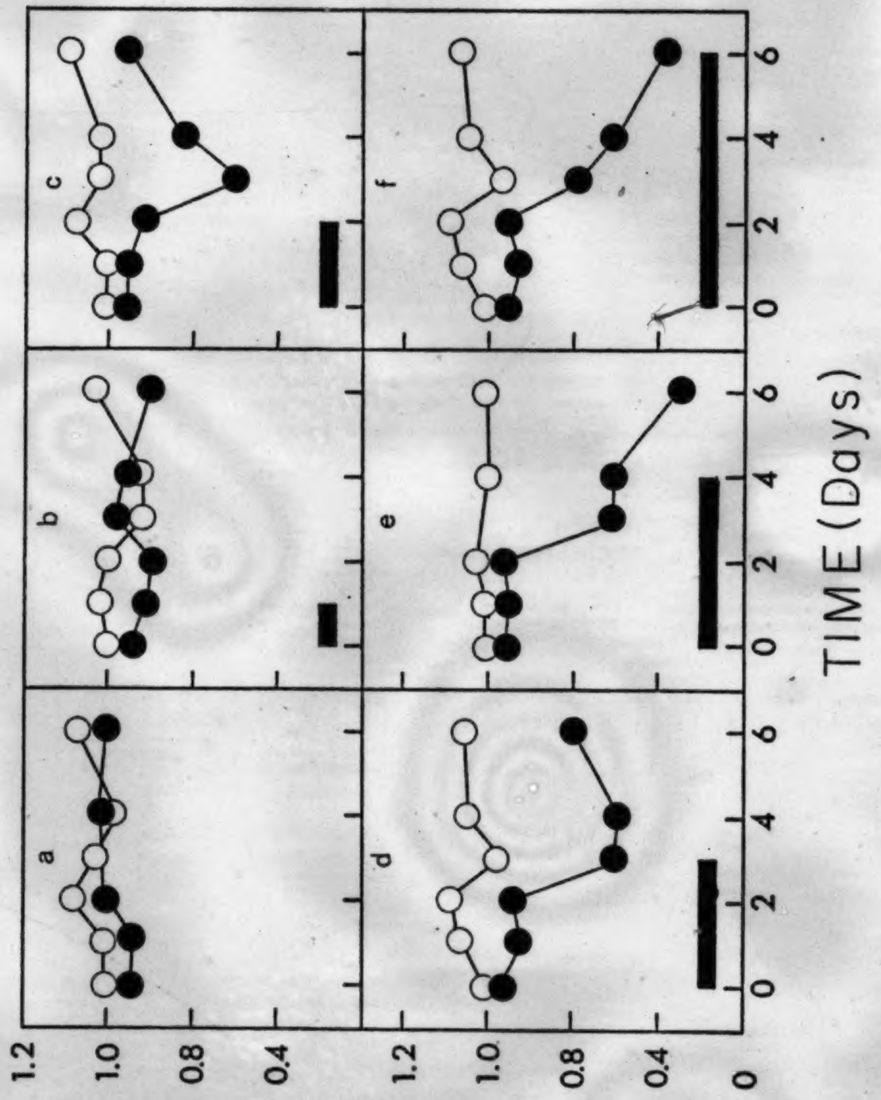
Expression of cold sensitivity is readily reversible up to 3 days of exposure (Fig. 2d). Recovery was marked by chlorotic leaves dying and falling off the plant while new

Figure 2

The effects of exposure to temperatures of 13°C for various times on the chlorophyll content of mutant and wild type *Arabidopsis*.

The chlorophyll content on a fresh weight basis of the wild type is indicated by open (O) symbols and that of the mutant by closed (●) symbols. The open and closed bars inserted in the figure indicate the times at which the plants were exposed to 22°C or 13°C, respectivly

Figure 2


a
b
c
d
e
f
1.2
1.0
0.8
0.4
0
0
2
4
6
TIME (Days)
CHLOROPHYLL CONTENT (µg mg fwt-1)

growth occurs at the apical meristem. Recovery does not appear to be inhibited by light or to need a dark period as seen in some cold stressed plants (Martin *et al*, 1981). Mutant plants exposed to 4 days or more of chilling temperature do not recover.

Photosynthesis And Respiration

As an early step in determining the nature of the defect in PM11, the magnitude of gas exchange by the mutant under light and dark conditions was compared to that of the wild type. The objective was to see if a cold induced change in photosynthesis or dark respiration could be oberved. A conditional defect closely related to photosynthesis or dark respiration should have an almost immediate effect on gas exchange of PM11 under non-permissive conditions. Examples of such defects have been shown in some photorespiratory mutants. A mutant deficient in phosphoglycotate phosphatase activity showed altered net CO_2 exchange after only 7 minutes under non-permissive conditions (Somerville and Ogen 1979).

PM11 was capable of wild type levels of photosynthesis and dark respiration under normal atmospheric conditions at both 22°C (Fig. 3) and 13°C (Fig. 4) for at least one hour. This suggests that the defect is not closely related to the metabolism involved with photosynthesis and dark respiration.

Because of the strongly injurious effects of the cold (death by 4 days), and the observation that cold stress in

Figure 3

Net CO_2 Exchange of wild type and mutant *Arabidopsis thalania* at 22°C

Net CO_2 exchange measurements were made at 22°C in 21% O_2,323 ul l^{-1} CO_2 , balanced N_2 at 350 uEinstiens $m^{-2}s^{-1}$. The values represent the mean of measurements on 3 separate plants. The broken line(----) represent the response of the mutant(PM 11) and the solid line(-) represents the wild type response. The positive values on the dependent axis represents CO_2 given off into the system(respiration) The bar inserted in the figure represents the time of illumination.

Figure 3

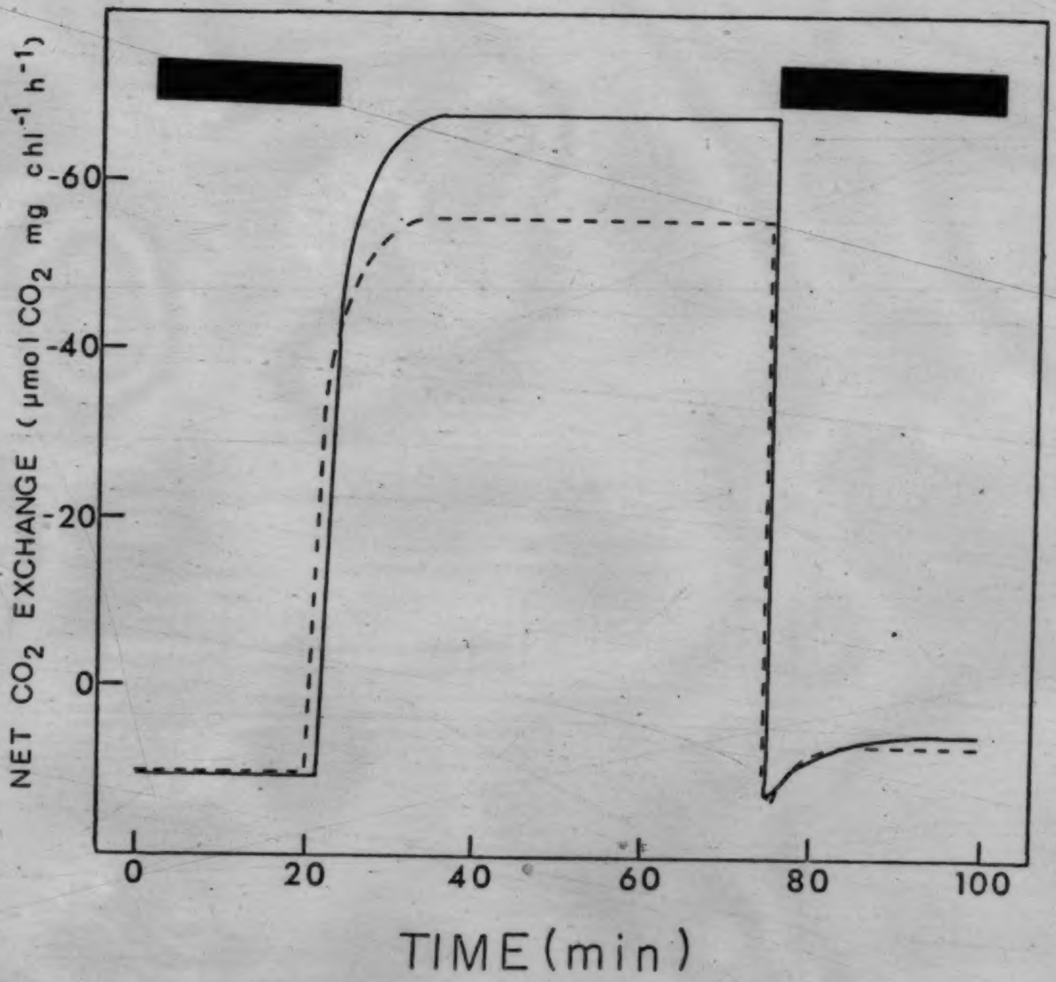
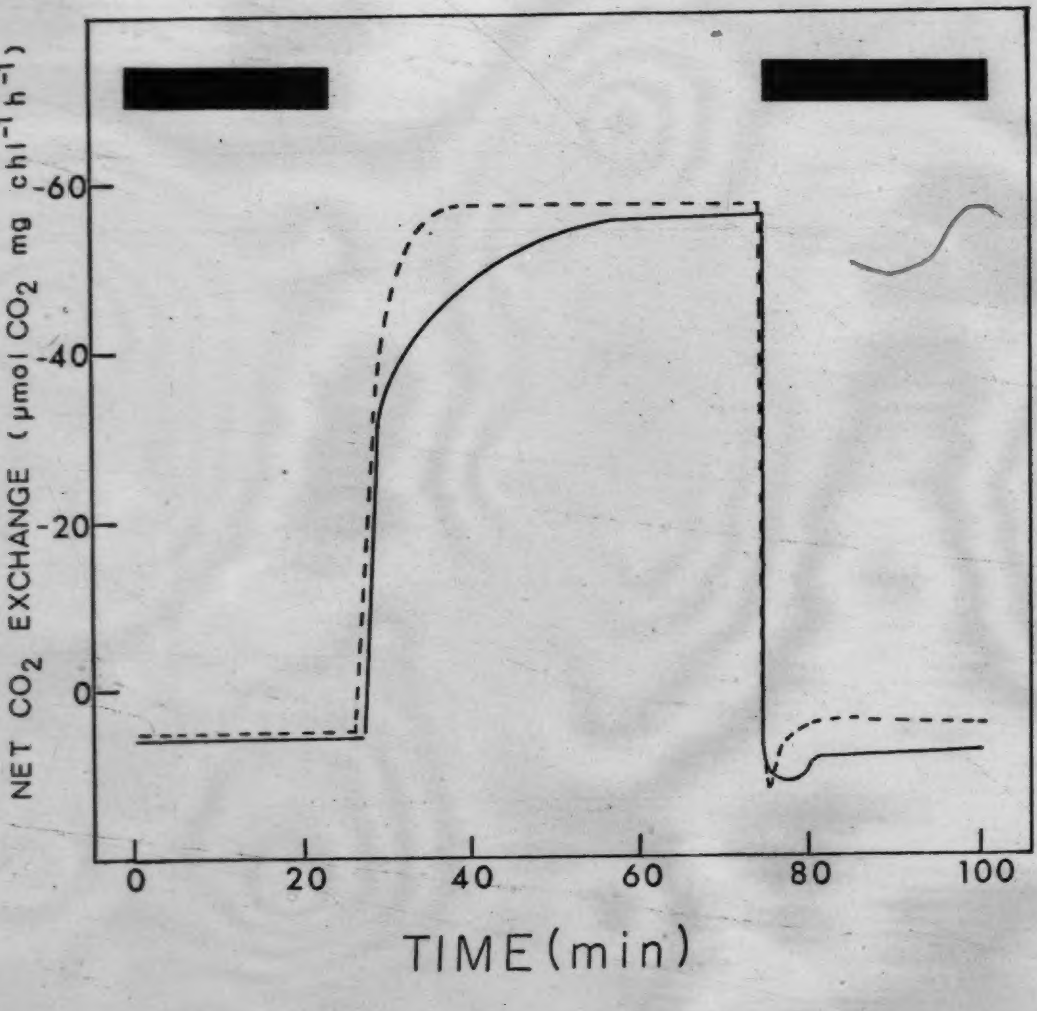

NET CO$_2$ EXCHANGE (µmol CO$_2$ mg chl^{-1} h^{-1})
-60
-40
-20
0
0
20
40
60
80
100
TIME(min)

Figure 4

Net CO_2 Exchange of wild type and mutant *Arabidopsis thalahia* at 13°C

Net CO_2 exchange measurements were made at 22°C in 21% O_2,323 ul l^{-1} CO_2 , balanced N_2 at 350 uEinstiens $m^{-2}s^{-1}$. The values represent the mean of measurements on 3 separate plants. The broken line(----) represent the response of the mutant(PM 11) and the solid line(-) represents the wild type response. The positive values on the dependent axis represents CO_2 given off into the system(respiration) The bar inserted in the figure represents the time of illumination.

Figure 4


NET CO_2 EXCHANGE (μmol CO_2 mg $chl^{-1} h^{-1}$)
-60
-40
-20
0
0
20
40
60
80
100
TIME (min)

cold sensitive plants is a slow progressive effect, it was considered potentially informative to monitor the photosynthetic and dark respiration rates in plants exposed to longer periods of chilling. Plants were transferred to 13°C and at 24 hour intervals 20 minute measurements of both photosynthesis and dark respiration were taken at 22°C. The results of this experiment are shown in Figure 5. After 24 hours at 13°C the mutant had about 80% of the wild type photosynthetic rate. By the second day of exposure to 13°C, mutants rates were reduced to about 50% of wild type. The decrease was progressive so that by 3 days of exposure to low temperature the mutant rate was about 10% of the wild type rate.

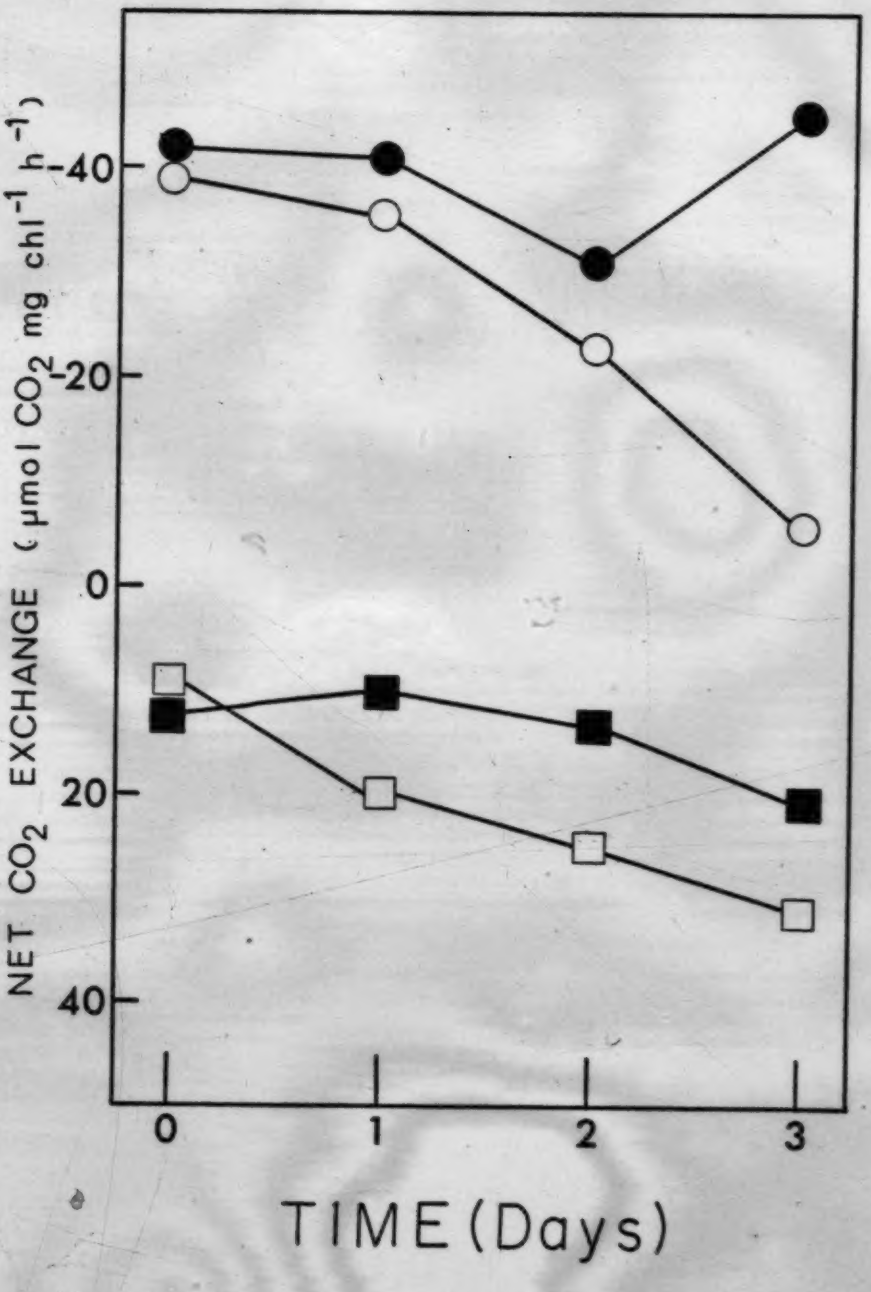
In parallel with these experiments, measurements of dark respiration in PM11 and wild type plants were also made (Fig. 5). Dark respiration decreased slightly in the wild type after one day of chilling but gradually increased after 3 days in the cold to about 1.8 times the prechilling level. In contrast to the wild type, after one day of chilling PM11 showed a dramatic increase in dark respiration to a rate twice that of the wild type. This increase continued at an apparently linear rate over the duration of the experiment. Although a common feature of chilling sensitive species is a decrease in mitochondrial respiration on prolonged exposure to chilling temperature, some chilling sensitive plants such as *Episcia reptans* and *Xanthium* do show increases in respiration rates under chilling conditions (Wilson 1979,

Figure 5

Effect of Exposure to Low Temperature on Photosynthesis and Dark respiration by Wild Type and Mutant *Arabidopsis.*

Wild type photosynthesis rate is indicated by closed (●) symbols and the mutant, by open (O) symbols. Positive values indicate greater dark respiration and negative values represent greater photosynthesis. Each point represents the mean of measurements on 3 plants. Plants were shifted to 13°C at time-0 and were removed at the indicated intervals for gas exchange measurements of 20 minutes duration in 308.8 l l^{-}, 21% O_2, balance N_2 at a flow rate of 100 ml. min^{-1}, 350 uEinsteins m^{-2} sec$^{-\ 2}$ 24°

Figure 5


NET CO2 EXCHANGE (μmol CO2 mg chl-1 h-1)
-40
-20
0
20
40
0
1
2
3
TIME (Days)

Drake and Raschke 1974). This has provoked the suggestion that the high respiration rate, observed in response to chilling, is due to peroxidation of membranes and general permeability of all membranes (Wilson 1979).

The large increase in dark respiration after one day of chilling, by contrast with the relatively slight effect on photosynthesis during the same time interval, suggests that the chilling was affecting dark respiration earlier and to a greater extent than photosynthesis. Therefore, the temperature sensitive phenotype of PM11 was tested to see if it was light independent. Plants were placed in the dark at 13°C and 22°C for 2 days, removed to the light for 12 hours and then chlorophyll content was measured on a fresh weight basis. The results show a decrease in chlorophyll content in PM11 as compared to wild type in plants exposed to 13°C in darkness, but no differential effect of dark treatment at 22°C (Table 1). Thus light is not required for the deleterious effect of exposure to cold on PM11.

Photosynthetic Electron Transport

Although previous experiments suggested that light is not required for the deterious effects of exposure to the cold on PM11, photosynthesis was nevertheless strongly affected. The decrease in photosynthesis observed after 2 days of chilling in PM11 could be due to depression of the light or dark reaction of photosynthesis. The light dependent reactions of photosynthesis take place on the thylakoid membranes which generate reducing power (NADP) and

TABLE 1

The effect of chilling temperature on chlorophyll content of mutant and wild type *Arabidopis* in the dark.

Strain	Temperature	
	22 °C	13 °C
WT	1.00	0.93
PM11	1.13	0.62

Wild type and PM11 plants were incubated for 2 days in darkness at 13°C and 22°C. Following the cold treatment plant were illuminated for 12 hours before chlorophyll determination. Values are expressed in ug chl mg fwt^{-1} and are the average of measurements of three plants.

energy (ATP) via electron transport and photophosphylation. The dark reactions are those of the Calvin cycle which use NADPH and ATP to fix CO_2.

As suggested earlier, the high respiration rate of PM11 upon exposure to chilling could be due to peroxidation of lipid membranes. Since photosynthetic reaction centres proteins and electron transport proteins are located in the choroplast membranes, changes in the membranes might be expected to affect the activity of the photosynthetic electron transport chain. In order to distinguish whether depressed phtosynthesis in PM11 was due to alterations in the light reactions, electron transport rates were measured in normal and cold stressed plants at various times after exposure of intact plants to chilling temperature (13°C).

Overall electron transport ability by broken chloroplasts of PM11 showed no inhibitory response, as measured by O_2 evolution, when compared to wild type until after 3 days of exposure to low temperature (Table 2). In contrast, photosynthesis as measured by net CO_2 gas exchange on intact plants had already declined 50% by this time (Figure 5).

It, therefore, seems that the early effect of the cold on photosynthesis is not mediated by a depression in the light reactions of photosynthesis. Furthermore, the high respiration rate seen in PM11 after one day exposure to chilling temperature is probably not due to peroxidation of chloroplast membranes since it would be expected to also

TABLE 2

Effect of exposure of intact mutant and wild type plants to chilling temperature on photosynthetic electron transport by isolated broken chloroplasts.

Time(hours)	Reaction	Strain	
		WT	PM11
12	Full	163.9	133.6
	PSI	308.9	291.1
	PSII	157.6	143.2
36	Full	118.9	135.3
	PSI	312.6	416.5
	PSII	136.6	104.4
60	Full	149.3	147.7
	PSI	292.6	289.2
	PSII	119.4	129.2
84	Full	109.5	29.1
	PSI	218.9	20.5
	PSII	98.5	18.4

Rates of electron transport as expressed as umoles of O_2 consumed or evolved mg chl^{-1} h^{-1}. Plants were exposed to 13°C temperatures for the indicated times before thylokoids were extracted and assayed at 25°C as described in Materials and Methods.

affect the electron transport chain and reaction centres which are associated with the thylakoid membranes.

The decrease in electron transport rates observed after 3 days of chilling was not localized to either photosystem I or II. Independent assays of both systems showed the two activities decline in parallel (Table 2).

It, therefore,appears that the depressed photosynthetic levels that are observed in the CO_2 gas exchange experiments are either due to direct inhibition of the dark reactions or indirect inhibition of some function related to CO_2 fixation.

$^{14}CO_2$ Labelling Analysis

The previous experiments suggested that the metabolism of PM11 was altered by chilling temperatures and that these effects are progressive. Furthermore the alterations appear to be related to dark respiration and to a lesser degree to affect the dark reactions of photosynthesis. A potentially informative method of studying these alterations was to examine the distribution of radioactive label in compounds extracted from plants allowed to photosynthesize in $^{14}CO_2$ under various conditions and for various lengths of time.

On the basis of gas exchange results, two time periods were used for the labelling studies. A labelling period of 15 minutes after one hour of preillumination at 13°C was chosen for detailed analysis of the early products of $^{14}CO_2$ fixation. This time period corresponds to the intial phase when photosynthesis rates of PM11 were very similar to those of wild type (Fig. 2). The purpose of such a labelling was to determine if any intermediate metabolite was accumulating or missing after a short period of stress. Since gas exchange data is only a gross measurement of the physiological state of the plant, it is possible that the effects of an accumulation or absence of a compound might not be detected over short periods of time. The distribution of products from the short term labelling is presented in Table 3. As was implied from CO_2 gas exchange the metabolism of PM11 appears to be normal following short (1 hour) exposure to non permissive conditions. Carbon appears to flow normally into the Calvin cycle and glycolytic pathways (Acid-2, Acid-3 functions), citric acid cycle intermediates (Acid-1), amino acids (Basics) and sugars (neutrals). Abnormal accumulation or depletion of a specific metabolite or class of metabolites was not observed. The insoluble fraction which is primarily starch is slightly higher in PM11. Unfortunately this fraction is difficult to quantify and the observation was not persued further.

These observations in conjunction with the gas exchange data suggested that the effects of chilling on PM11 were not closely related to photosynthesis. Hence a second labelling period was chosen which would radioactively label a broader range of compounds such as fatty acids. Such compounds are not primary products of photosynthesis but alteration in their metabolism might explain the reduction in photosynthesis.

TABLE 3

Distribution of products of 15 min of ^{14}CO assimilation by mutant and wild type *Arabidopsis* at chilling temperature.

Fraction	------- Strain ------ WT	PM11
Basics	34.9	37.5
Glutamate	8.2	9.4
Glycine	5.0	4.8
Alanine	6.9	7.3
Serine	11.5	12.0
Neutral	20.6	22.6
Acid 1	21.6	18.0
Citrate	3.1	2.4
Malate	6.7	5.9
Glycolate	2.3	1.9
Acid 2	13.7	11.4
Acid 3	7.3	8.0
Insolubles	1.7	3.1
Recovery	98.8	100.6

Plants were illuminated for 1 hour at 13°C and 350 ueinsteins $m^{-2}s^{-1}$ before labeling with $^{14}CO_2$ for 15 minutes at the same conditions. Values presented are the percentage of total labeled carbon found in the soluble and insoluble fractions. The gas regime was 308 ul l^{-1}, CO_2, 21% O_2, balance N_2. Wild type and PM11 fixed 50.7 and 54.4 umoles of CO mg chl^{-1} hr^{-1}, respectively.

To ensure the distribution of $^{14}CO_2$ label into as many metabolic pools as possible, plants were labelled for 24 hours at 13°C. Gas exchange measurements demonstrated that chilling effects on photosynthesis are not dramatic in PM11 until after a 24 hour exposure to the cold. Therefore, to ensure the metabolism of PM11 would be under stress at the time of labelling the plants were shifted to 13°C for one day before labelling commenced. Thus, the products isolated at the termination of labelling were representative of plants that had been stressed for 2 days at 13° C.

The results of the 24 hour labelling experiment showed little differences in the distribution of labelled products at the level of resolution afforded by comparisons of percentage distribution in the ion exchange fractions (Table 4). Approximately 50% of the radioactive label was found in the neutral fraction in both wild type and PM11. Upon further separation by thin layer chromotography the label was equally distributed between 4 neutral sugar compounds. On the basis of Rf values and comparision with standards 3 sugars were identified as sucrose, fructose and glucose (Plate II). The other sugar has the Rf value expected of ribose. These results support the contention that the photosynthetic apparatus is functioning normally since sucrose is the major end product of photosynthesis. Also, although photosynthesis is declining in the mutant by this time (2 days of chilling), the overall percentage of product distribution is not effected. This is consistant with an

TABLE 4

Distribution of products of 24 hours of $^{14}CO_2$ assimilation by mutant and wild type *Arabidopsis* at chilling temperature.

Fraction	------- Strain ------	
	WT	PM11
Basics	4.4	5.7
Neutral	46.7	47.7
Acid	10.4	12.5
Non polar	19.2	19.7
Insolubles	6.7	6.9
Recovery(%total)	87.4	92.5

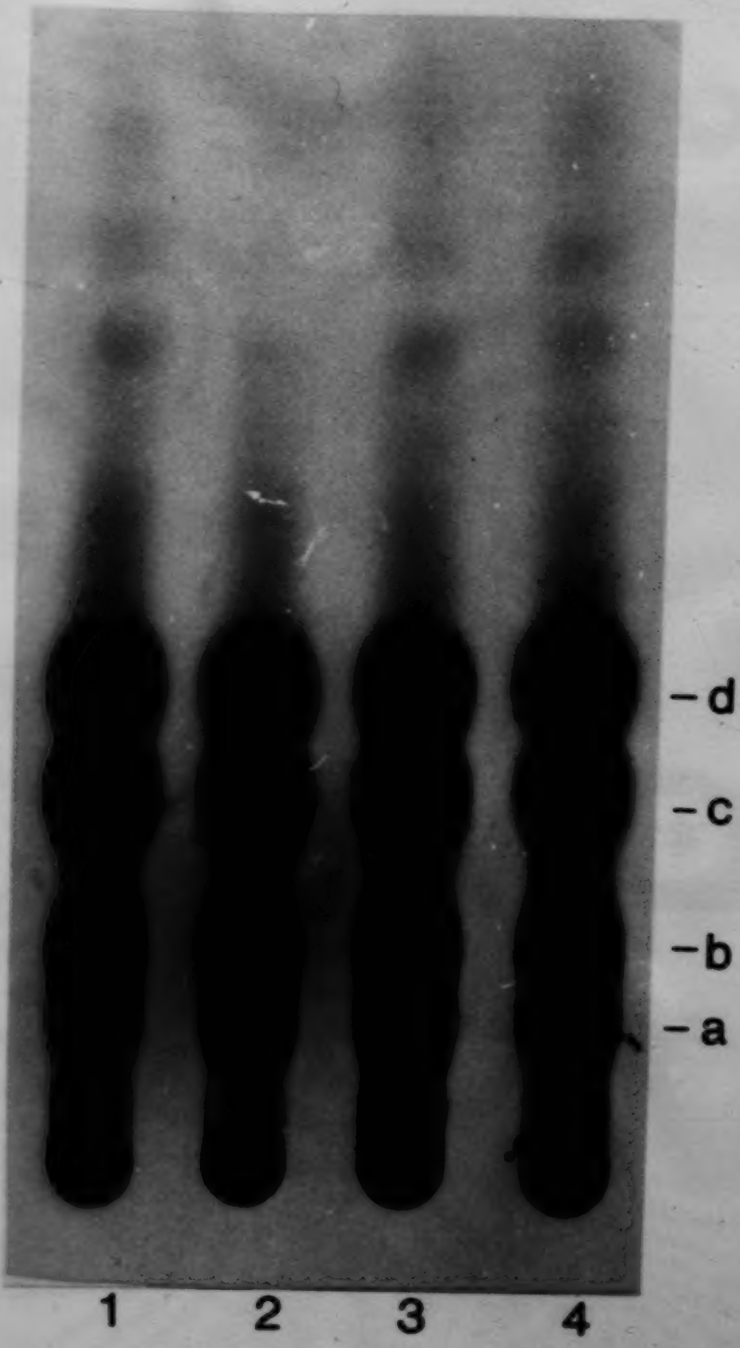
Plants were shifted to 13°C for 24 hours then labeled for 24 hours at 13°C with 350 ueinsteins $m^{-2}s_{-1}$ of illumination. The gas regime was 308 ul l^{-1} CO_2 , 21% O_2, balance N_2. Eight plants were labeled (4 wild type:4 mutant) and 2 plants of each type were pooled for each sample. On average the wild type fixed 35 umoles CO_2 mg $chl^{-1}h^{-1}$ and PM11 fixed 30.4 umoles CO_2 mg $chl^{-1}h^{-1}$ Values are given as percentage of total carbon fixed in the soluble, insoluble, and organic fractions.

Plate II

TLC separation of neutral sugars from 24 hr labelling at 13°C of wild type and mutant *Arabidopsis thaliana*

Lanes 1 and 2 are products from wild type and lanes 3 and 4 are from mutant PM 11. Compounds are:(a)Unknown (b)Sucrose; (c)Glucose; (d)Fructose; The same number of counts were applied to each track.

Plate II



effect on the overall process rather than a specific effect on a particular part (enzyme) of that process.

The acid fraction which contains the organic acids (TCA intermediates), sugar monophosphates and sugar diphosphates also show approximately the same distribution in mutant and wild type. Components of this fraction were separated by TLC using various solvent systems suited for resolution of particular compounds. A solvent system, diethyl ether: acetic acid:water (65:15:5) which preferentially separates organic acids showed no major differences in compound distribution (Plate III). The majority of label was found in compounds which comigrated with glycolate and malate, with lesser amounts found in citrate. The radioactivity which remains at the origin is sugar monophosphate and diphosphates. Separation of the sugar mono and diphosphates proved difficult. A number of 2 dimensional solvent systems were employed but all gave inconsistent separations. The best resolution was afforded by the system described by Crowley(1963). Fractionation of the acid fraction from the wild type and mutant in this system revealed no convincing differences.

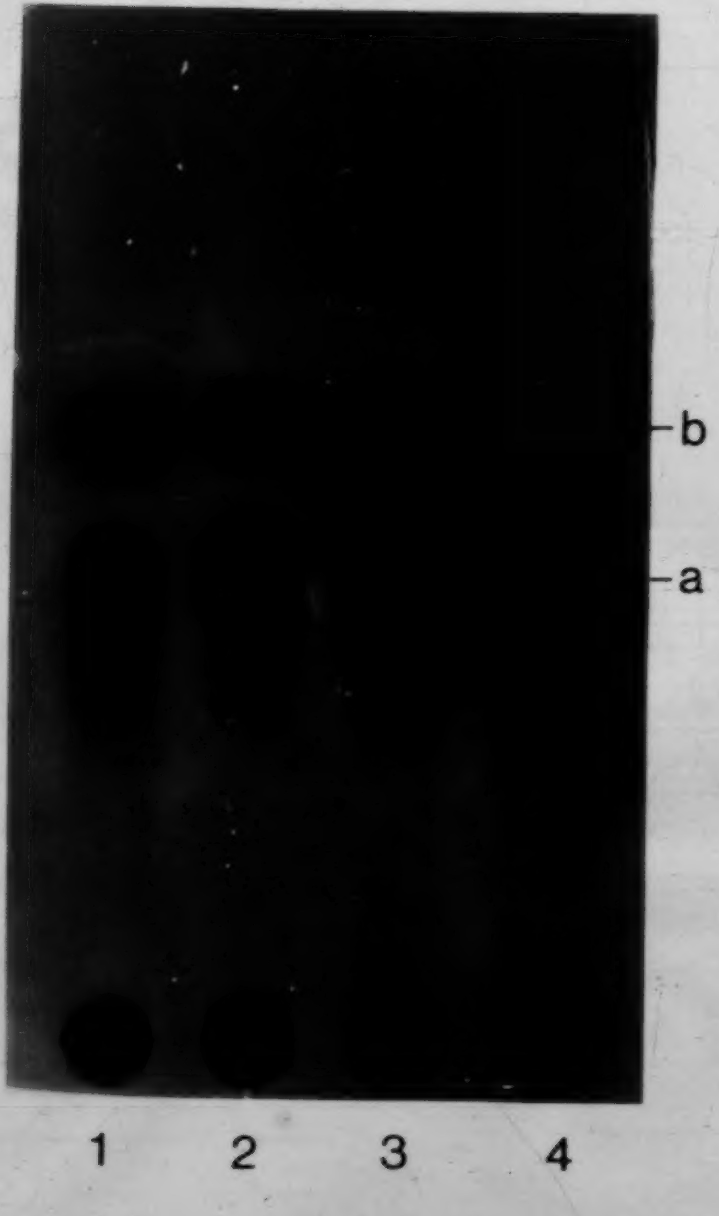
The basic fraction, which contains the amino acids, made up approximately 5% of the total labeled products in both mutant and wild type after 24 hours. The compounds in this fraction were well separated using a 2 dimensional solvent system (Plate IV). There appear to be 17 major labeled compounds which were tentatively identified by

Plate III

TLC separation of organic acids from 24 hr. labelling at 13°C of wild type and mutant *Arabidopsis thaliana*

Lanes 1 and 2 are products from wild type and lanes 3 and 4 are from mutant PM 11. Compounds are: (a)Malate and citrate; (b)glycolate and possible 2-oxoglutarate .

Plate III

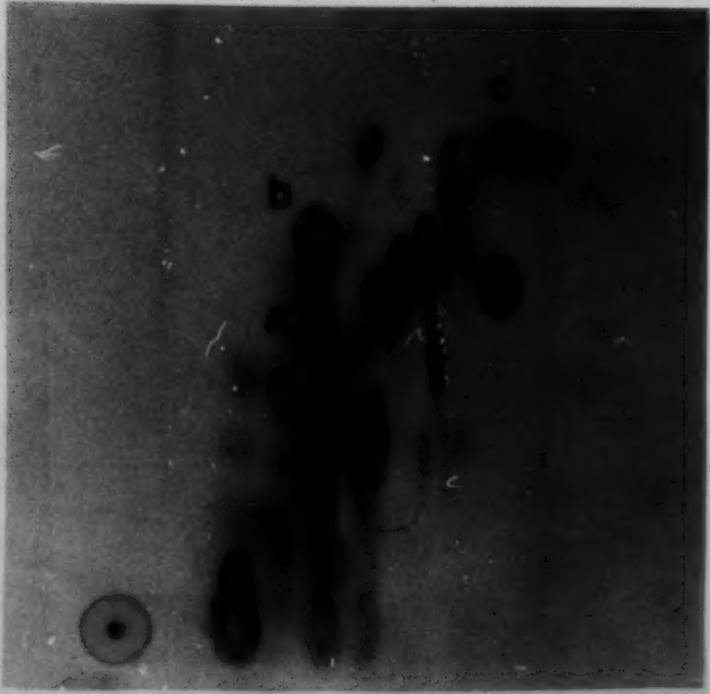


' Plate IV

TLC separation of basics (amino acids) from 24 hr labelling at 13°C of wild type and mutant *Arabidopsis thaliana*

The upper autoradiogram is of a separation of wild type products and the lower is of products of the mutant PM11. The first dimension was from bottom to top of the page. Compounds:(a)glycine; (b)serine; (c)phenylalanine.

Plate IV



published Rf values for the solvent system. Differences could by detected in 3 compounds. These are believed to be serine, glycine and phenyalanine.

Serine is present in about a 2-fold excess in PM11 as compared to wild type with a slight reduction seen in glycine levels. A possible explanation for the altered glycine/serine levels could be the speed at which the plants were killed after their removal from the $^{14}CO_2$ atmosphere. The experimental design did not allow for rapid killing of the plants. Because the rate of flux of carbon through photorespiration is quite high, this would cause inconsistencies in the labelling patterns of photorespiratory intermediates such as serine and glycine.

The increase in phenylalanine could be due to an increase in phenolic compounds which is a common response of plants to stress. The major precursor of these phenolic compounds is phenylalanine. Since PM11 is under cold stress there could be an increased requirement for phenylalanine. Aside from these 3 compounds no major differences could be detected between PM11 and wild type with respect to amino acid metabolism.

The insoluble fraction contained about 7% of the total radioactive label in both mutant and wild type. The similarity suggests that starch metabolism of PM11 is not affected by the cold.

Overall, the major metabolic pathways of photosynthesis, dark respiration, amino acid metabolism, and starch metabolism all appear to be normal in terms of product distribution. This suggests that there are no enzymatic lesions in these metabolic pathways which are responsible for the chilling sensivity of PM11. However, the *colD1* mutation causes dramatic effects on the overall rates of photosynthesis and dark respiration even though it does not appear directly related to those processes.

The fraction which showed the most dramatic changes was the non polar fraction. This group of compounds which are soluble in chloroform made up approximately 19% of the radioactive label in both PM11 and wild type. The majority of these compound are considered to be lipids and pigments.

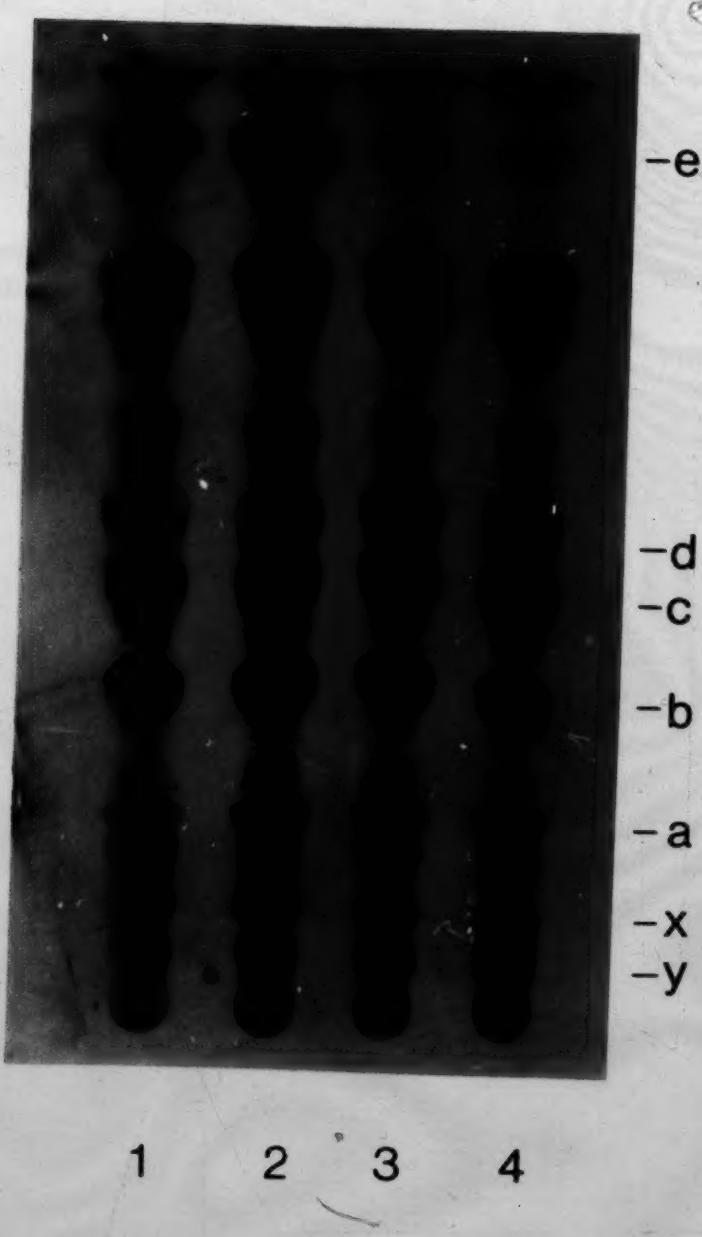
The non polar fraction was separated into individual compounds using a number of 1 and 2 dimensional solvent systems. The first solvent system separated polar lipids such as phospholipids and sulfolipids on the basis of the head group attached to the three position of the glycerol moiety. Separation by this system showed one major difference in distribution of radioactivity (Compound-E, Plate V). The mutant accumulated this compound in large excess to that in wild type extracts. Unfortunately this compound is not one of the common phospholipids since it did not co-migrate with the lipid standards used. The high mobility in this solvent system suggests it is neutral or only weakly charged with respect to the glycerol head groups. Since the extact identity of the majority of spots resolved by this and other solvent systems for separating

Plate V

Separations of polar lipids fraction from a 24 hr. labelling experiment of wild type and mutant *Arabidopsis* at 13°C

Lanes 1 and 2 are mutant and lane 3 and 4 are wild type PM 11. Compounds are: (a)Phosphatidyinositol; (b)Phosphatidylcholine; (c)Phosphatidylgycerol and Phosphatidyethanolamine (e)Unknown; (x)Unknown; (y)Unknown.

Plate V


-e
-d
-c
-b
-a
-x
-y
1
2
3
4

lipids was not determined, Letters have been assigned for convenience. The difference in intensity of spot-E represented the first major biochemical difference detected between wild type and PM11. It was, therefore, decided to study the polar lipid distribution of PM11 and wild type from this 24 hour labelling at 13°C in more detail. The compounds in the non polar fraction were fractionated with greater resolution by using a two dimensional separation which employed the same solvent system used in Plate V for the first dimension and a more acidic system for the second dimension (Nichols and James 1966). The results are presented in Plate VI. As before, a major difference in amount of label in spot-E between mutant and wild type could by detected, but at least 5 other less pronouced differences were also apparent. Spots-F and G, readily detected in PM11, are less intense or missing altogether in the wild type, The opposite is true of spot-I and J which are present in the wild type and absent in the mutant.

To determine any other differences in the non polar fraction, this fraction was separated using a solvent system which preferentially separates neutral lipids. The polar or charged compounds stay at or close to the origin. The results are presented on Plate VII. Four spots were detected which differed in amount between wild type and PM11 (spots K,L,M,N). In all cases the spots were more intense in the mutant versus the wild type. Spot K and N show the most marked difference between wild type and PM11 with

Plate VI

Two dimensional separation of non polar compounds (lipids and pigments) from 24 hr labelling of wild type and mutant *Arabidopsis* at 13°C

The lower autoradiogram is of a separation of wild type products and the upper is the products of the mutant PM 11. The first dimension was from left to right and the second dimension was from bottom to top of the page. Letters indicates differences between wild type and mutant PM11.

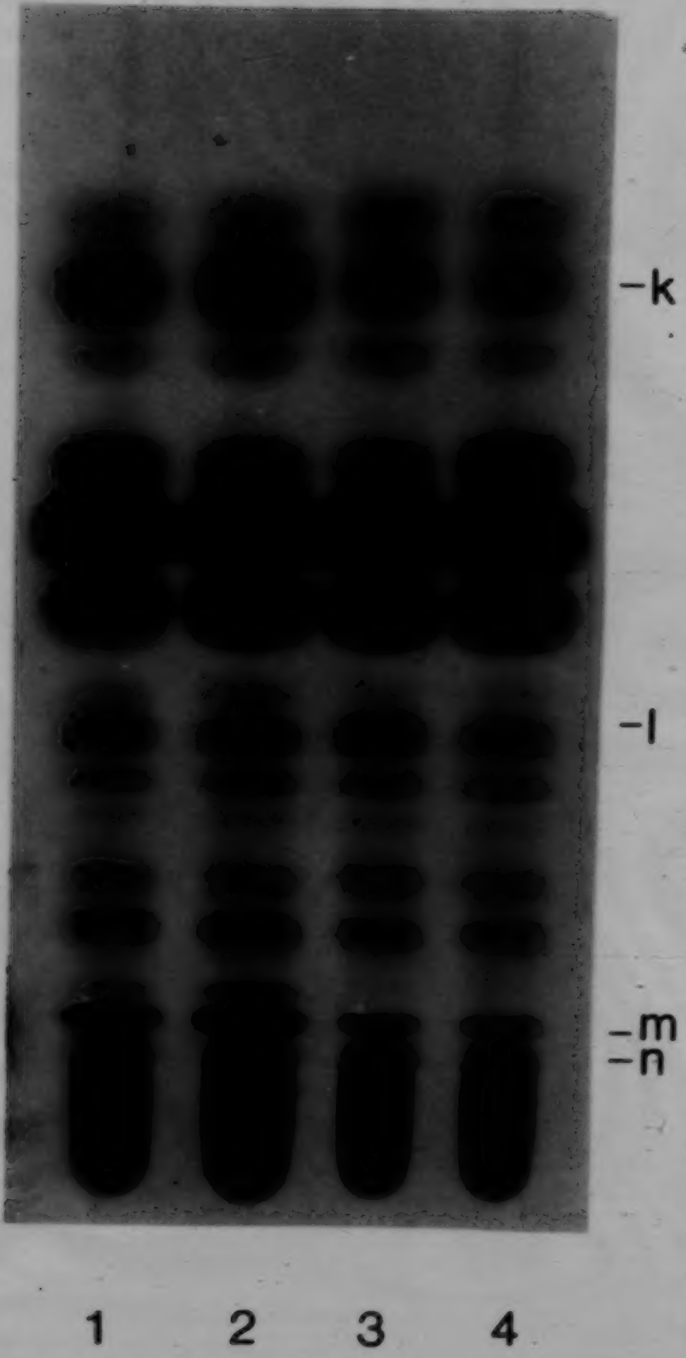
Plate VI

Plate VII

Separation of neutral lipid and pigments from a 24 hr labelling of wild type and mutant *Arabidopsis* at 13°C.

Lanes 1 and 2 are wild type and lanes 3 and 4 are from mutant PM 11. Letters (k,l,m,n) represent differences between wild type and mutant.

Plate VII



approximately a 10 and 2-fold difference respectively in amount over the wild type. The intensity of spots L and M is only slightly different in wild type and mutant, but is sufficient to be visually detected. Spots were not quantified in any of these experiments so overall differences could not be established. What could be established from these experiments was that there appears to be at least 1 major and 9 minor differences in the labelling pattern of the wild type and PM11 under stressed condition (13°C). More importantly this is the only fraction from the 24 hour labelling experiment which shows a major difference.

The next question was to determine if these differences were temperature dependent. If the alteration in distribution is due to a temperature sensitive enzyme, one would expect the differences to disappear if the same experiment was carried out at 22°C. On the other hand, if the labelling pattern remained the same in the mutant, irrespective of whether it was labeled at 22°C or 13°C it would suggest that the defect is not conditional but the *effects* of the defect on plant viability are.

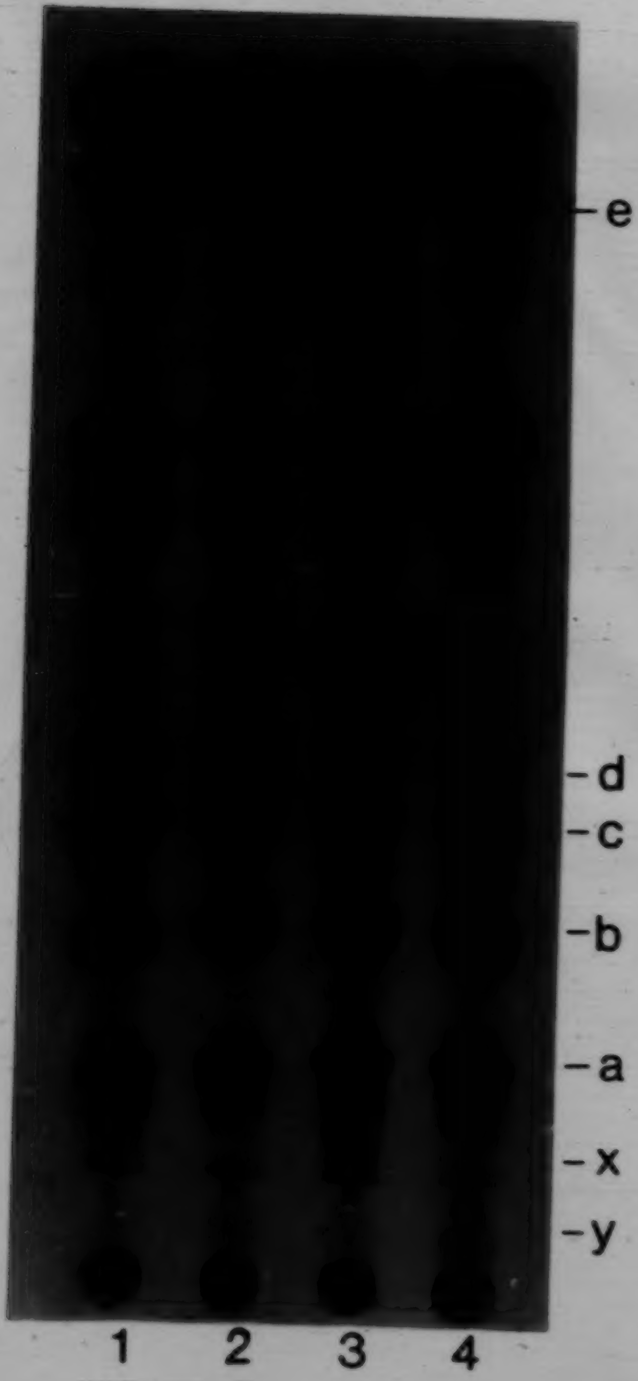
The mutant and wild type were therefore labeled for 24 hours with $^{14}CO_2$ under exactly the same conditions as the previous experiments except the temperature was maintained at 22°C. The phospholipids and pigments were extracted and separated by one dimensional thin layer chromotography as before (Plate VIII). The results show that the distribution of radioactivity appears to be similar in PM11 and wild type

Plate VIII

Separation of polar lipids fraction from a 24 hr labelling experiment of wild type and mutant *Arabidopsis* at 22°C

Lanes 1 and 2 are wild type and lane 3 and 4 are mutant PM 11. Compounds are: (a)Phosphatidylinositol; (b)Phosphatidylcholine; (c)Phosphatidylgycerol and Phosphatidyethanolamine (e)Unknown; (x)Unknown; (y)Unknown.

Plate VIII



e
d
c
b
a
x
y
1
2
3
4

under these conditions. In particular, spotE appears to be present in the same relative amount in both wild type and PM11. The intensity of spot-E in the wild type at 22°C is similar to that found in PM11 after exposure to 13°C for 2 days. The implication is that the metabolism of this compound changes in the wild type but not in the mutant in response to a shift to colder temperatures. Also, spots X and Y which are of low mobility in the phospholipid solvent system are prevalent in both wild type and PM11 at 13°C but are not seen at 22°C in either of these strains. The presence of these spots may be related to an adaptive physiological response to low temperature or may reflect some uncontrolled variable in the preparation of the samples.

^{14}C-Acetate Metabolism

The previous section on long term labelling experiments suggested that the only significant alterations between PM11 and wild type was in the fraction which contained non polar compounds such as pigments and lipids. The major drawback of doing a long term $^{14}CO_2$ labelling experiment, in the present context, is the lack of specificity of which compounds one can label. As a further step to verifying an alteration in lipid metabolism, leaves of both wild type and PM11 were labeled *in vivo* with ^{14}Cacetate, a relatively specific precursor or lipid biosynthesis. Previous studies have demonstrated that ^{14}C-acetate is readily incorporated into leaf fatty acids (Slack and Roughan, 1975). In these studies, when expanded spinach leaves were fed ^{14}C-acetate they incorporated label into the acyl groups of typical membrane lipids phosphatidylcholine (45%), phosphatidylglycerol (20%) and diacylgalactosylglycerol (15%). Thus, the use of acetate allows prefential labelling of the lipids, which was not possible with $^{14}CO_2$.

Another drawback of the 24 hour labelling experiment is the relatively long time period required to ensure penetration of $^{14}CO_2$ into the fatty acid pool. Because of the long labelling period, both photosynthesis and dark respiration are abnormal in PM11 by the completion of the experiment. This could be of significance as it has been demonstrated that isolated chloroplasts will only synthesize fatty acids at rates similar to those obtained with intact leaves *in vivo* if acetate is used as a precursor. The implication is that acetate derived from leaf mitochodia is the physiological fatty acid precursor (Murphy and Leech, 1981). Since both PM11 mitochondria and chloroplast fractions are altered by 2 days of chilling it might not seem unusual to see alterations in lipid metabolism. In other words, one cannot be sure whether the altered lipid metabolism is causing chilling sensitivity or is a result of chilling sensitivity. $^{14}CO_2$ acetate labelling afforded an opportunity to approach this problem more directly since relatively short pulse - chase experiments could be performed.

There are many possible variations of pulse-chase experiments. The procedure used was to label the plants under permissive conditions at 22°C for 12 hours to allow complete saturation of as many fatty acid pools as possible. Since the labelling is at permissive temperatures, the acetate should be metabolized normally. Plants were then shifted to non permissive temperatures (13°C), samples taken 12, 24, and 48 hours after the shift and the metabolites compared to samples taken at time 0 (before the shift). These time points represent the range of PM11 metabolism from normal (time 0) to drastically altered (48 hours). The twelve hours to 24 hour period is when mitochondial respiration begins to increase; the 24 to 48 hour period is when photosynthesis begins to decline (Fig. 3). A minor difference detected at the early times might be the cause of major changes in the metabolism of the mutant which become apparent later . The results of this experiment are presented on Table 5. The percentage of radioactivity in the six most prominent compounds was quantitated by eluting spots from TLC plates and counting by liquid scintillation counting. Since none of the compounds have been unequivocally identified, they have been assigned numbers in Table 5. An autoradiogram of the TLC separation is presented as Plate IX.

The metabolism of ^{14}C-acetate under permissive temperatures (22°C) is similar in the mutant and wild type except for a slight increase of 2% (total incorporation) in

TABLE 5

^{14}C - Acetate labeling of leaves of wild type and mutant *Arabidopsis in vivo*

Time	Strain	Compound a	b	c	d	u	v
0	WT	4.2	26.1	29.1	31.5	3.1	5.9
	PM11	4.3	23.9	28.4	32.5	3.1	8.3
12	WT	3.6	26.8	24.9	35.2	3.5	5.7
	PM11	5.3	27.5	24.8	29.3	2.8	10.2
24	WT	4.3	25.0	22.1	33.4	3.2	8.9
	PM11	5.5	26.6	27.2	17.4	2.4	17.9
48	WT	3.7	27.3	20.1	26.0	4.6	14.4
	PM11	4.2	25.3	18.0	20.4	4.6	22.7

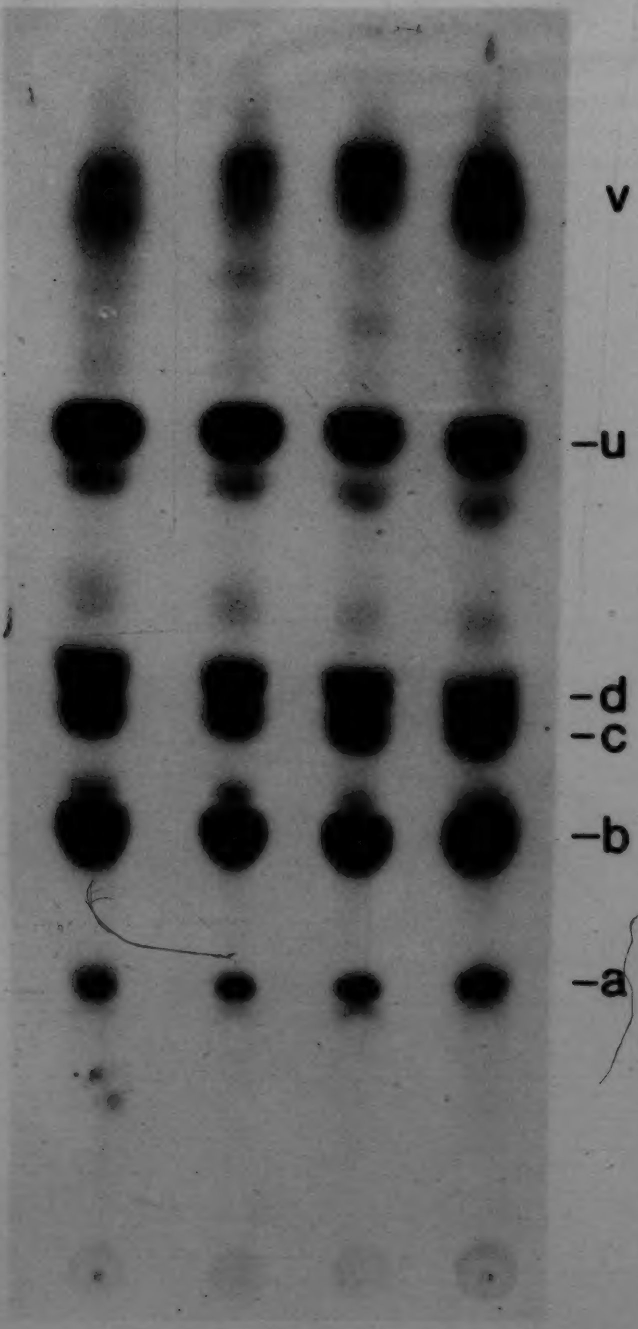
Plants were labeled with ^{14}C-acetate for 12 hours at 22° C by application of label to leaves. The leaves were then washed and the plants transfered to 13°C with continuous illumination. At the indicated times after the completion of the labelling, samples were taken for product analysis as described in Materials and Methods. The values in the table represent the percentage of the total incorporation into chloroform - soluble compounds. The compound numbers correspond to those on Plate X.

Plate IX

Separation of non polar lipid fraction from the ^{14}C-Acetate labelling experiment of wild type and mutant *Arabidopsis* at 13°C

Lanes 1 and 2 are wild type and lane 3 and 4 are mutant PM 11. Compounds are: (a)Phosphatidylinositol; (b)Phosphatidylcholine; (c)Phosphatidylgycerol and (d)Phosphatidyethanolamine (u)Unknown; (v)Unknown;

Plate IX



compound V in the mutant. Compound V represents both compound E and the neutral lipids and pigments seen in the 24 hour $^{14}CO_2$ experiment. Exposure of plants to 13°C causes a dramatic increase (3 fold) in the amount of label in compound V in the mutant, and a substantially less dramatic but consistent increase in the amount of label in this compound in the wild type. This supports the previous observation from $^{14}CO_2$ labelling which showed differential incorporation of label in this compound in the mutant and wild type after a 48 hour exposure to 13°C.

However, the increase of label in compound V in the wild type following exposure to 13°C is in contrast with the $^{14}CO_2$ labelling results which showed a large decrease in compound V in the wild type as the temperature was lowered. One possibility is that compound V in the ^{14}C - acetate labelling represents a different group of compounds than that seen in the $^{14}CO_2$ experiments.

The increase in compound V in PM11 is offset by a decrease in compound U as compared to wild type. The levels of compound U appear to be similar in wild type and mutant at time 0, but the mutant shows a steady decrease to 50% of the levels seen in wild type by 24 hours of chilling. This decrease tapers off at the 48 hour stage but there is still a substantial difference. All other compounds (A,B,C and D) remain relatively constant.

To summarize, the increase in compound V seen in PM11 is gradual and correlated with a decrease in the amount of label in compound U. More importantly, the changes in lipid metabolism can be detected at the 12 hour stage, well before photosynthesis begins to decline and just before dark respiration increases, implying it might be a cause of these two effects rather than a result.

Compounds A and B appear to be phosphatidylinositol and phosphatidylcholine on the basis of co-migration of standards. Compound C and D probably represent a mixture of phosphatidylethanolamine and phosphatidylglycerol. The wild type exhibits three major changes in lipid metabolism in response to a shift to cold temperature. Compound C and D show decreases of 30% and 16%, respectively, while compound V shows an increase of 3 fold. This suggests the wild type is acclimating to the chilling temperatures in a time dependent fashion. It is important to note that 2 of the 3 compounds which change in response to chilling in the wild type show alterations as compared to wild type in the mutant. These results are consistent with the results from $^{14}CO_2$ labelling which suggested that adaption to chilling involves altered lipid metabolism.

Gas Chromatography of Fatty Acids

The ability to withstand chilling injury in both porkiothermic animals and chilling sensitive plants has been associated with the degree of saturation of the fatty acids of the membranes (Lyon and Raison 1970). The higher the degree of unsaturation, the lower the temperature at which the phase change occurs in the lipid portion of the membrane. Evidence for the importance of lipid unsaturation is inferred in part from observed differences following exposure of many species to reduced temperature. For example, the transfer of *Brassica napus* to 5°C leads to increases in lenolenic (18:3) acid in roots and leaves. This increase is most evident in the phosphatidylcholine and ethanolamine fraction of leaves and in neutral lipids and an undentified phospholipid of the roots (Smolnska and Kuiper, 1977). However, there is some doubt as to whether the degree of unsaturation of the fatty acids plays a dominant role in determining the temperature at which the phase change occurs in the lipids of cellular membranes. Analysis of *Passiflora* species which vary in their sensitivity to chilling revealed that pronounced differences in chilling sensitivity of the *Passiflora* species can be correlated with physical differences in their membrane lipids. However, the degree of unsaturation of the lipids varied only slightly and was not a reliable guide to chilling sensitivity (Patterson *et al*, 1978). Furthermore the addition of cholesterol to synthetic phospholipids has been shown to lower the temperature of transition until, at a third mol amount the transition vanishes (Willemot 1980). Therefore, it appears the sterols could play an important role in determining the temperature at which phase trasitions occur in membranes and that the degree of unsaturation of the fatty acids might not be as imporatant as it was once considered.

In view of these considerations the fatty acid saturation levels of PM11 and wild type were examined. The ^{14}C labelling of wild type and PM11 has shown alterations in lipid metabolism in the mutant. Changes in the levels of various lipid classes also should affect the total fatty acid composition of the plant, since each lipid class does not contain the same proportion of the different fatty acids. Therefore PM11 and wild type plants grown at 22°C and shifted to 13°C for 2 and 4 days were analysed for fatty acid composition and degree of saturation by gas chromatography. The results are presented in Table 6. The fatty acid composition of the 8 major identifiable peaks revealed no major consistent or convincing differences between mutant and wild type. One peak of an undentified compound (#3) was substantially higher in wild type than in mutant, however this peak was of very variable amount as indicated by the high standard diviation and cannot be considered a convincing difference. A composite value for the degree of unsaturation has been described by Dogras *et al*, (1977). Calculation of the value for wild type and mutant *Arabidopsis* revealed no difference (Table 7). In contrast to expectation the ratio of unsaturated to saturated fatty acid decreased about 6% during the four day chilling period in both PM11 and wild type. From these results it appears that the effect of chilling on PM11 is not primarily in the degree of unsaturation. The difference in proportion of fatty acid UK 3 may bear reinvestigation.

TABLE 6

Fatty acid composition of wild type and mutant Arabidopsis

		Peak	22°C		13°C		13°C	
Compound		Number	WT Day0	PM11	WT Day2	PM11	WT Day4	PM11
	UK#1	1	2.10±0.23	1.75±0.37	2.92±0.97	2.62±0.14	.37±0.03	.70±0.42
	UK#2	2	1.40±0.14	1.88±0.28	1.49±0.01	1.40±0.07	1.34±0.16	1.49±0.20
	UK#3	3	8.38±4.49	2.18±0.43	2.54±0.05	1.76±0.88	10.24±5.68	1.71±0.38
Palmitic	16:0	4	13.30±0.98	14.68±0.02	15.46±1.97	14.74±0.71	12.83±0.05	14.09±0.69
Palmitoleic	16:1	5	2.95±0.32	2.72±0.38	3.03±0.12	2.87±0.04	2.28±0.14	1.99±0.07
	UK#4	6	8.26±0.61	9.71±0.34	8.32±0.05	8.46±0.31	8.15±1.61	8.10±0.41
Stearic	18:0	7	1.22±0.07	0.87±0.23	1.42±0.71	1.93±1.16	1.74±0.74	1.32±0.42
Oleic	18:1	8	2.74±0.44	2.99±0.34	2.88±0.67	4.39±2.71	2.55±0.41	3.11±0.51
Linoleic	18:2	9	10.07±0.24	11.86±0.46	10.02±0.71	11.49±2.13	8.51±0.12	10.60±1.41
Linolenic	18:3	10	44.79±3.15	48.48±4.96	47.66±2.10	46.39±6.56	44.28±5.66	43.31±2.43
	20:0	11	0.33±0.16	0.35±0.12	----	----	0.44±0.10	0.23±0.05
	20:1	12	0.55±0.27	0.28±0.18	----	----	----	----
	UK#5	13	1.75±0.39	1.19±0.08	----	----	0.84±0.04	1.18±0.16

Plants grown at 22°C were shifted to 13°C and the indicated intervals samples were taken for fatty acid separation by GLC as described in Materials and Methods. The values represent the mean value for three plants. The peak numbers in the table correspond to those in Figure 6.

TABLE 7

Ratio of unsaturated to saturated major fatty acids of wild type and mutant *Arabidopsis* following exposure to chilling temperature.

Strain	Time of exposure to chilling temperature 0 hours	48 hours	96 hours
WT	4.17	3.91	3. 95
PM11	4.25	3.91	3. 75

The ratio of unsaturated to saturated fatty acids was calculated from the data in Table 6 by the formula of Dogras *et al* (1977)

$$\frac{\text{Unsaturated}}{\text{Saturated}} = \frac{\%16{:}1 + \%18{:}1 + \%18{:}2 + \%18{:}3}{\%16{:}0 + \%18{:}0}$$

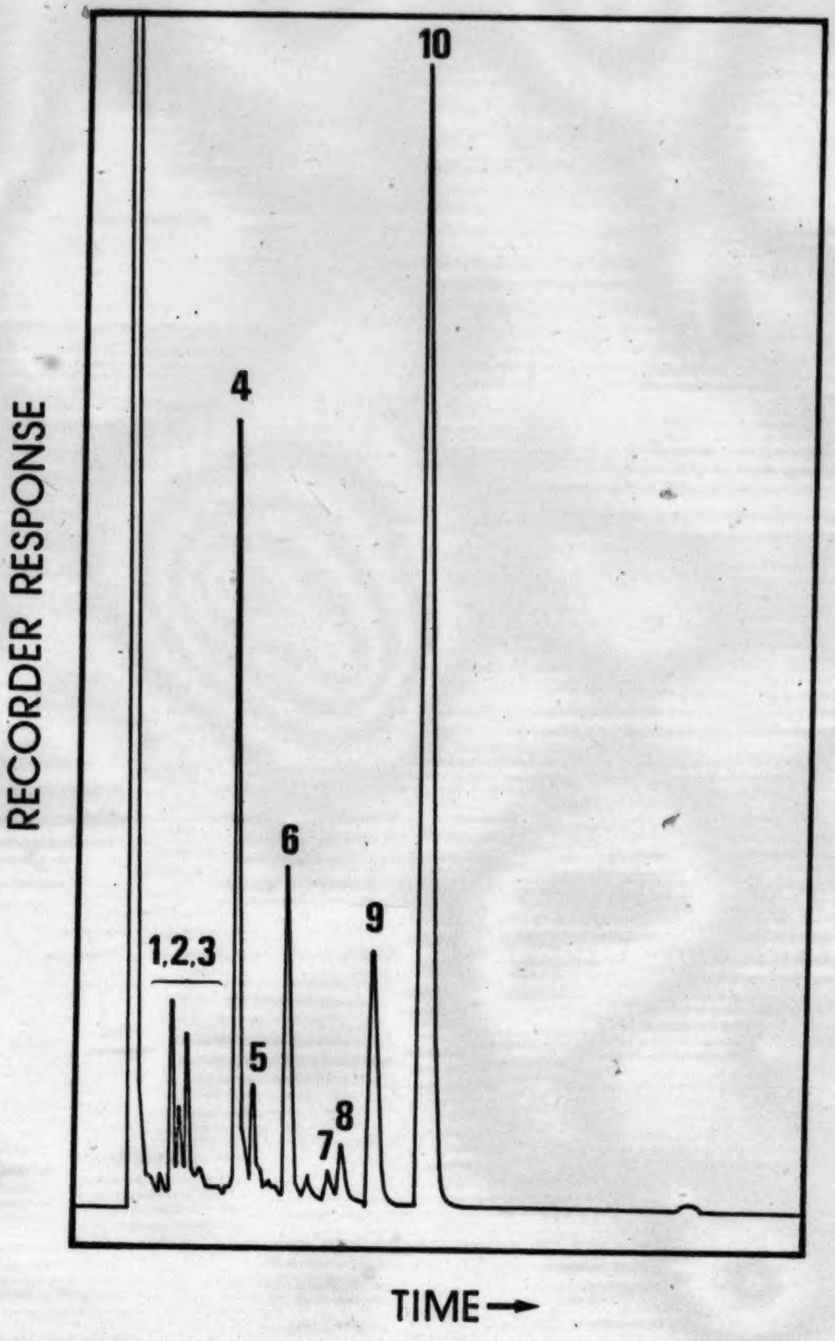
This compound appeared to fluctuate in the wild type upon exposure to chilling from 8% to 2.5% back to 10% of the total fatty acid composition during the four days of exposure to cold temperature. The amount in PM11 remained relatively constant at approximately 2% of the total fatty acid composition. Unknown 3 was later found to have the same retention time on the GLC as 14:0 suggesting it is myristate. Variability of Unknown 3 within samples of the same day might also suggest it is an artifact of the isolation procedure. The remaining unknown peaks (1, 2, 4 and 5) all appear similar in the mutant and wild type. A typical tracing of the fatty acid composition of wild type and PM11 is presented as Figure 6.

Figure 6

Gas Chromatography tracing of wild type *Arabidopsis*

Typical seperation of fatty acids from *Arabidopsis* by gas chromatography. The numbers(#) correspond to Table V.

Figure 6



IV. DISCUSSION

During the past 10 years many reports have appeared describing numerous events associated with cold stress in higher plants. These events range from physiological effects on photosynthesis and dark respiration to changes in membrane fluidity. However, in spite of the many studies, a complete understanding of what constitutes low temperature resistance, or how temperature results in injury, is lacking. A systematic study of a simple system or mechanism usually involves direct measurement of its components and factors that directly effect that mechanism. For more complex systems such as temperature stress, which appears to involve many unknown components, a different approach is necessary. This entails identifying each unknown component by altering that component so that it changes the response of the complete system to external stimuli. In principle, this facilitates identification of the component and its function by physiological and analytical comparison of the two systems which differ only in that response. The specific objective of this study, was to test this approach. The idea was to examine the possibility that a complex mechanism such as chilling resistance could be dissected one component at a time by the isolation of mutants which have lost chilling resistance.

The approach depends on the undemonstrated assumption that at least some components of the resistance mechanism are dispensable at permissive temperatures. Mutants with alterations in some component of the resistance mechanism would be chilling temperature sensitive and therefore non viable in non-freezing cold temperatures. A major conceptual problem with the method is lack of specificity in the screening procedure. How does one distinguish a mutant with a temperature sensitive protein from a mutation which makes a normally chilling tolerant plant chilling sensitive? In principle, the most straightforward approach would be to demonstrate the presence of a metabolic or structural alteration which is expressed at both permissive and restrictive temperatures. Subsequent demonstration that the alteration confers chilling sensitivity can be achieved by showing restoration of the alteration in selection of revertants resistant to chilling temperatures.

In contrast, if the cold sensitivity of the mutant is due to a temperature sensitive enzyme, the mutant would be expected to be structurally and metabolically similar to wild type at permissive but not restrictive temperatures. Unfortunately these criteria provide no clue as to what kind of difference one might expect between the mutant and wild type. The initial experimental approach was, therefore, designed to assay general areas of metabolism at a gross level. These measurements would hopefully provide information on the areas of metabolism that were affected by the mutation. This approach was then followed by introducing ^{14}C - label into the major metabolic pools in the hope of indentifying an alteration at the metabolite level.

Intuitively one would expect a temperature sensitive enzyme to show an altered metabolite level in one or a group of closely related compounds. For a mutation in a mechanism involved in chilling sensitivity it is difficult to predict the biochemical alteration but one might expect the phenotype of the mutant to mimic many of the physiological responses to cold seen normally in cold sensitive species. The three main physiological responses of a chilling sensitive plant are 1)altered dark respiration rate, 2)progressive loss of photosynthesis and 3)leakage of ions and electrolytes from tissue (Lyon *et al*, 1979).

A mutant was isolated that is sensitive to chilling temperatures (13 - 16°C). The mutation responsible for this effect shows mendelian segregation and has been tentatively designated the *colD1* locus.

Biochemical Characterization

Because of the general effects of the *colD1* phenotype a number of parameters had to be determined before detailed biochemical analysis could be intiated. These were 1) the time frame needed under non permissive conditions to see a physiological change, 2) the nature of the changes whether progressive or sudden and 3) which areas of plant metabolism are effected initially. The temperature shift (Fig. 1) and gas exchange results (Figs. 3 and 4) suggest the lethal effects of the *colD1* mutation at chilling temperatures are progressive and cannot be measured until after 24 hours of stress. The lack of measurable effect on photosynthesis and dark repiration under short term (1 hour) chilling conditions (Figs. 2 and 3) implies that the *colD1* gene product is not a temperature sensitive enzyme closely related to either of these functions. Electron transport studies (Table 2) show that the reduction of photosynthesis by 48 hours is not due to a dissruption of the light reactions which appear normal at this time. Verification that the *colD1* mutational effects on photosynthesis are secondary was shown by the lethal effects of this mutation under dark conditions at 13°C (Table I). The loss of chlorophyll probably reflects the reduced photosynthetic rates after 3 days of exposure to 13° C. The conclusions drawn from these experiments at the gross level are that the *colD1* mutation effects dark respiration, photosynthesis, and chlorophyll synthesis indirectly. However, dark respiration and the dark reactions of photosynthesis are effected in a progressive manner and these alterations probably contribute to the lethal effect of the mutation.

Although these experiments helped in establishing a time frame in which to carry out physiological measurements of the plant under stressed conditions, they were relatively uninformative in terms of the function of the *colD1* gene. The level of complexity and resolution of analysis was increased by labelling the major metabolic pools of the mutant under permissive and non permissive conditions with $^{14}C_2$-label. Crude analysis of the distribution of $^{14}C_2$-label in the various classes of compounds revealed no striking difference in amino acid or carbon metabolism (Plates II, III, IV) in short term or long term labelling times at 13° C. However, an alteration in the metabolism of non polar compounds such as lipids and pigments was detected in the *colD1* mutant exposed to 48 hours of chilling conditions (Plate V, VI, VII). One major change in labeled compounds (spot-E) and 9 minor changes could be detected. Further analysis with ^{14}C - acetate, which preferentially labels lipids (Murphy and Leech, 1981; Slack and Rougham, 1975) demonstrated an increase in one compound (spot V) and a decrease in another (spot-U) relative to wild type (Table V). The changes reached maximal differences after 24 hours. Unfortunately the identify of these compounds has not been determined. They are believed to be phospholipids since the solvent systems used to separate both $^{14}CO_2$, and ^{14}C-acetate non polar fractions were specific for phospholipids.

The onset of detectable changes (12 hours) in conjunction with the lack of any other noticable differences in labeled metabolite levels suggests that the *colD1* mutation directly affects lipid metabolism. The changes in labelling distribution could be due to an actual enzymatic defect in lipid metabolism or a defect in some other pathway of metabolism which indirectly causes a disruption in lipid metabolism. Evidence against this latter hypothesis is inferred from the lack of altered metabolism in any other area as assayed by $^{14}CO_2$ labelling at 13°C.

The increased dark respiration rate in the mutant after exposure to 13°C is similar to the observations in other chilling sensitive plants such as *Episica reptans* and *Xanthium strumarium* (Wilson 1979; Drake and Raschke 1974). Peroxidation of disintegrating membrane components and the general permeability of all membranes has been suggested as a possible reason for this phenomenom in these plant species (Graham and Patterson, 1982). Although this is a possibility in the *colD1* mutant, this effect would be expected to disrupt not only lipid metabolism but also other metabolic functions quite early after onset. The results of this study are insufficient to explain the increase in CO_2 evolution in the dark following chilling.

The alterations in lipid metabolism are not reflected in altered saturation/unsaturation levels in the *colD1* mutant suggesting that changes at the total lipid pool level are small and cannot be detected by gas chromotography of total leaf fatty acids. However, the level of saturation does not appear to play a major role in providing chilling resistance to *Arabidopsis* since the ratio of unsaturation to saturation does not increase in the wild type response to chilling (Table 7).

It appears, therefore, that the primary effect of the *colD1* mutation is disruption of normal lipid metabolism. The complexity of the altered labelling pattern reflects the elaborate pathway by which lipids are synthesized (Rougham and Slack 1982 for review). A major step in resolving the nature of the defect will be to identify the compounds which have changed and determine how they relate to each other from known pathway descriptions. These determination will be complicated since each spot revealed by TLC may contain a range of compounds which share a common polar head group. Therefore, each spot will have to be separated into its constituents by GC analysis.

The *colD1* gene product appears to play a role in adjusting lipid metabolism to chilling conditions. Upon examination of the wild type $^{14}CO_2$ labelling patterns at 22°C and 13°C differences in 3 compounds (spot X, Y, E) can be detected. The reduction in spot E and the disappearance of spot X and Y could be due to a response of wild type *Arabidopsis* to chilling temperature. This acclimation process does not appear to occur in the *colD1* mutant for spot-E. In contrast to the wild type, the amount of label entering this compound in the mutant is approximately the same at either temperature and is similar to the amount seen in the wild type at 22° C but not 13°C (Plate V and VII). This suggests that a possible effect of the *colD1* mutation is an alteration in the plants ability to adjust its lipid metabolism in response to changes in temperature. Further support for this hypothesis comes from the pulse - chase experiments performed with ^{14}C - aetate (Table 5). Compounds C, U and V show a change in distribution in response to chilling in the wild type. However, compounds U and V in the *colD1* mutant show an altered response relative to wild type.

Therefore, 3 of 6 phospholipids which appear to adjust in response to chilling as discerned by $^{14}CO_2$ and ^{14}C - acetate labelling studies show altered distribution levels in the mutant at cold temperature. However, caution must be used in interpreting changes in labelling patterns of pathways due to the direct effects of temperature on enzyme kinetics. The rate of most enzymatic functions doubles with each 10°C increase in temperature (Q_{10} = 2.0). These temperature coefficients vary between enzymes depending on the activation energy of the catalysed reaction. The changes in lipid distribution may only be a response of enzymes of these pathways to differential temperature and bear no relationship to an acclimation mechanism.

System Analysis

It is difficult to assess the potential usefulness of this genetic approach to analysing the mechanisms of temperature stress in higher plants because the results are only preliminary. Isolation and further characterization of a spectrum of mutants might reveal patterns of defects which are prevalent and therefore provide a better insight into whether the system is worth pursuing. The frequency of cold sensitive mutants in an EMS treated M2 population of *Arabidopsis* is about 10^{-4}. In contrast, mutations affecting photorespiration arise at a frequency of 10^{-3} (Somerville and Ogren, 1979). This suggests a limited number of mechanisms for confering a cold temperature sensitive phenotype to *Arabidopsis*. While the chemical basis of loss of function of a protein at high temperature is understood (ie. weakening of chemical bonds maintaining the structure of the protein, resulting in denaturation), mechanisms for loss of function at low temperature are less readily apparent. The reconstitution of ribosomes *in vitro* takes place by a reaction which is dependent on temperature. Therefore, it has been argued that mutations which affect assembly processes might be detected at cold temperature. However, aside from a few exceptions like ribosomal mutants the effects of chilling on protein structure are not well understood.

There is no readily available experimental evidence to indicate that proteins undergo conformational changes over a narrow range of temperatures. However, it has been suggested that solidification of membrance lipids at low temperature may cause changes in the configuration of membrane bound enzymes and transporters (Lyon 1979). Major support for this theory is derived from the corrolation of loss of many protein functions with changes in membrane fluidity (Lyon 1979 for review). The general consensus is that plants which can maintain membrane fluidity at low temperature are *chilling tolerant* whereas plants that cannot are *chilling sensitive*. It is particularly interesting therefore, that the one mutant isolated by the criteria of chilling sensitivity is defective in lipid metabolism.

The *colD1* mutation confers on *Arabidopsis* many of the characteristics of a typical chilling sensitive species.

Dark respiration increases in response to chilling are seen in a number of chilling sensitive plants (*E. reptans, X. strumanium*). Photosynthesis shows a slow progressive decline which is typical of almost all chilling sensitive plants. The *colD1* mutation also confers drought sensitvity to the plant. If the *colD1* mutants are not watered twice a day they begin to yellow and grow poorly. The first symptoms of chilling injury in many agriculturally important species is wilting and the appearance of necrotic patches on the leaves. These observations have led investigators to suggest that the permeability of the plasmalemma to water and ions increases as a result of a phase transition in the membrane lipids (Wilson 1979). Wilson has also shown that certain chilling sensitive plant species (eg: *Phaseolis vulgaris*) can be chill hardened if exposed to 100% relative humidity at 5°C. The effect has been explained by the fact that stomates open in response to chilling and the water loss can be counteracted by the humid atmosphere. The cause of stomatal opening is unknown, but a phase change in the membranes of the guard cells could alter their permeability properties.

The effects of the *colD1* mutation on photosynthesis and dark respiration are progressive and occur later than the alteration in lipid metabolism. (Table 1 and III). Because the lipid changes occur before photosynthesis and other functions begin to decline this implies that the primary effect of the mutation is to alter lipid metabolism and that the other changes observed are secondary effects of stress.

An alteration in lipid composition whereby the lipid bilayer cannot remain fluid at 13°C will cause the membrane to solidify gradually due to its heterogenity. Small clusters of highly saturated lipids will be transformed into rigid aggregates followed by a steady rise in the size and number of solid phase domains. This gradual change would be characterized by a progressive loss of enzymatic function rather than a sudden decrease in activity.

This hypothesis suggests, therefore, that the minor changes seen in phospholipid content in the *colD1* mutant could be responsible for the loss of the ability to maintain fluid membranes. The role of phospholipid polar head groups in regulating the fluidity of membranes of higher plants has not been ascertained. However, a more that 30°C difference in transition temperature between pure phosphatidylcholine and phosphotidylethanolamine having the same saturation acyl chain compositon has been shown (Cronan, 1978). Other workers have shown that when dipalmityl and mysritate phosphatidylcholines (transition temperatures 41°C and 23°C respectively) were added to polar lipids from chilling resistant plants, a transition occured around 7 - 8 °C (Wright and Raison, 1981). Normally these polar lipids show no transition in response to chilling down to 0 - 2°C and only 1% of both additives were needed to induce this effect. This work suggests that transition to a solid state is co-operative in the membrane. When one or a small mixture of phopholipids begin to change from a liquid - crystalline state to gel state the other lipid species will follow. Therefore, the range of resistance to chilling temperatures in a plant could be dependent on the amounts of minor phospholipids existing in the membrane. The increase in some of the lipid compounds in the mutant could lead to a co-operative transition of the lipid membranes at a much higher temperature than seen in the wild type. For example, it may be envisioned that the wild type normally reduces the amount of certain lipid compounds in response to reduced temperature because they will cause a co-operative transition of all the phospholipids. It seems possible that the *colD1* mutant does not or cannot remove these compounds and therefore a phase transition takes place causing a chilling sensitive effect. The reason why the mutant cannot remove these compounds could be due to a defective enzyme or a defect in the ability to "turn on" this enzyme (ie.a defect in acclimation). Since temperature would be the regulator of such a system, mutants defective in induction will appear cold sensitive because of lack of induction leading to chilling injury.

The mechanism by which temperature could regulate such a process might be similar to the mechanism suggested for regulation of fatty acid desaturases in *Tetrahymena* (Thompson, 1979). Thompson and co-workers believe membrane fluidity itself is a regulating factor controlling the activity of the membrane bound fatty acid desaturases.

Membrane changes due to lipid phase changes result in displacement of proteins into new positions or conformations which enhance or depress activity.

A similar hypothesis might apply to the *colD1* gene product. This locus could encode a membrane embedded protein. Under normal circumstances when the membrane fluidity decreases the enzyme is brought into a more favourable configuration to carry out its function which is to remove or alter compounds in the membrane. A defective enzyme that cannot respond to temperature and, therefore, cannot carry out this function will cause the plant to become chilling sensitive.

Thus, it is apparent that although the approach described here has promise as a novel methodology, the possible complexity of the chilling response is likely to preclude the formulation of a simplistic all embracing theory.

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